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MASCULINE COPULATORY BEHAVIOR IN INTACT AND CASTRATED FEMALE RATS[1]

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CERTAIN FEMALE ANIMALS are known to display mating reactions characteristic of the male of their species. Behavior of this type has been observed in the case of female pigeons (1), night herons (2), rabbits (3, 4), guinea pigs (5), cats (8), pigs (7, 8), cattle (9, 10), monkeys (11, 12), and chimpanzees (13). Despite the wide spread character of such 'reversals' of sexual behavior, exhibition of masculine mating behavior by experimentally altered females is frequently offered as evidence to substantiate claims of induced sex reversal (14–19).

In the case of the female rat, masculine copulatory behavior has always been regarded as either nonexistent or quite rare (17, 20, 21–24). Previously the senior author (25) described 7 virgin female rats which mounted sexually-sluggish males. More recently two experienced females have been observed to exhibit the complete masculine copulatory pattern exclusive of the ejaculatory response (26). In all of these instances the females were in estrus and readily accepted the male in typical feminine fashion.

The present study was conducted to determine the proportion of female rats showing masculine copulatory behavior, the frequency of such reactions in each individual, and the relationship of masculine responses to the female's estrous cycle.

METHODS

Twenty virgin female rats, 3 to 6 months old, raised in segregation were tested for masculine and feminine copulatory behavior. Five animals were discarded before the completion of the experiment because they showed little or no male behavior; and the remaining 15 rats were given daily sex tests throughout 4 estrous cycles. Vaginal smears were analyzed before each test. The experimental female was allowed a short period of adaptation in the testing cage and then a sexually receptive female was introduced. At the end of 5 minutes the receptive female was removed. A vasectomized but sexually vigorous male was placed in the cage and left with the experimental female until he either copulated or attempted to copulate 3 times. The receptive female was re-introduced and the 3 rats remained together in the cage until 5 minutes from the introduction of the male. The male was then removed and the experimental and receptive females were observed together for another 5 minutes.

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TABLE 1. FREQUENCY WITH WHICH EACH FEMALE EXHIBITED THE THREE TYPES OF MASCULINE COPULATORY RESPONSES

Rat	Total Tests Given	Sexual Clasp				Palpation and Pelvic Thrusts				Complete Pattern			
		No. tests in which response appeared	% tests in which response appeared	Times response was executed	Average frequency of response per positive test	No. tests in which response appeared	% tests in which response appeared	Times response was executed	Average frequency of response per positive test	No. tests in which response appeared	% tests in which response appeared	Times response was executed	Average frequency of response per positive test
3	32	17	63	60	3.5	8	34	31	3.9	1	3	2	2.0
5	28	22	79	102	4.6	6	21	11	1.8	0	0	0	0
9	19	15	79	67	4.5	10	53	45	4.5	0	0	0	0
31	19	15	79	83	5.5	7	37	23	3.3	0	0	0	0
33	33	19	58	50	2.6	16	48	34	2.1	1	3	2	2.0
34	18	13	72	42	3.1	0	0	0	0	0	0	0	0
35	40	24	60	123	5.1	15	28	46	3.1	3	8	4	1.3
36	19	14	74	43	3.1	5	26	16	3.2	0	0	0	0
37	29	28	97	114	4.1	6	21	19	3.2	0	0	0	0
38	32	22	72	62	2.8	14	44	35	2.5	0	0	0	0
39	21	18	86	65	3.6	4	19	4	1.0	0	0	0	0
42	29	21	72	73	3.5	5	17	6	1.2	0	0	0	0
43	28	23	82	143	6.2	10	36	45	4.5	2	7	3	1.5
44	17	17	100	77	4.5	11	65	53	4.8	1	6	1	1.0
45	26	18	69	104	5.8	8	30	19	2.4	0	0	0	0
Total	390	286	1142	1208	62.7	125	479	387	41.5	8	27	12	7.8
Mean	26	19.1	76.1	80.5	4.2	8.9	34.2	27.6	3.0	1.6	5.4	2.4	1.6

The daily testing period for each rat thus consumed 15 minutes and there was ample opportunity to observe and record both the masculine and feminine mating behavior shown by the experimental animal.

The masculine copulatory behavior of the experimental females was divided into 3 types of responses: *a*) *reaction* 1, sexual clasp in which the mounting rat clasps a second animal with the forepaws about the lateral lumbar region; *b*) *reaction* 2, palpation with pelvic thrusts, which consists of mounting, palpating with forepaws and executing pelvic thrusts; and *c*) *reaction* 3, complete pattern which includes mounting, palpating and showing pelvic thrusts, giving a final forceful thrust and dismounting with a pronounced backward lunge. The complete pattern is frequently followed by licking of the genitals in a manner typical of the copulating male. In previously published studies of the male rat's mating behavior *reaction* 2 has been called an 'attempt' and *reaction* 3 has been referred to as a 'copulation.' A fourth response shown by males but not dealt with here is the 'plug reaction' which accompanies ejaculation.

RESULTS

Frequency of masculine responses. Certain elements of the masculine copulatory pattern are commonly exhibited by the majority of the female rats in our experimental

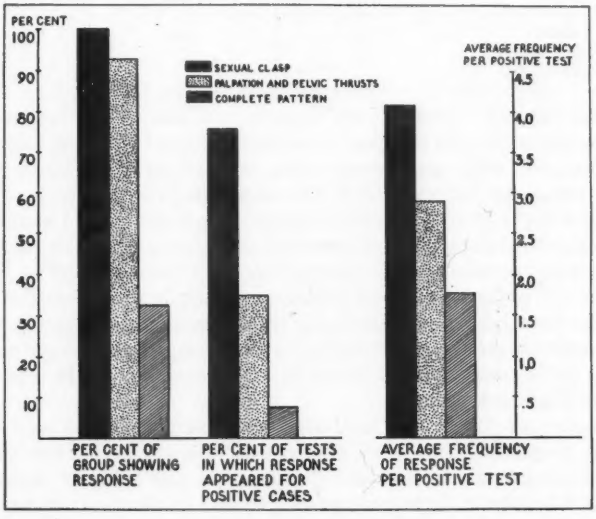


Fig. 1. Proportion of 15 females exhibiting each type of masculine response and average frequency of each reaction.

colony. Of 20 virgin females raised in segregation 18 executed the sexual clasp. Fifteen animals showed palpation and pelvic thrusts; 5 females displayed the complete copulatory response. Five cases which showed only the sexual clasp were not tested extensively. The remaining 15 females received from 17 to 40 tests, the results of which are summarized in table 1 and figure 1.

The sexual clasp, which was exhibited by each of the 15 animals, appeared in an average of 76 per cent of the tests. During those tests in which this response occurred it was executed an average of 4.2 times per test. Palpation with pelvic thrusts was shown by 14 females, and these animals exhibited this behavior in an average of 34

per cent of their tests. When the reaction was shown it occurred an average of 3 times per test. The complete copulatory pattern, executed by 5 of the 15 rats, was present in an average of 33 per cent of their tests. During those tests in which it appeared, the complete pattern was displayed on an average of 1.6 times per test.

Effects of repeated testing. The high percentage of females exhibiting masculine mating reactions, and the frequency with which such responses could be elicited in the animals studied were difficult to reconcile with the general agreement of earlier workers that this form of behavior is rare in the rat. Furthermore, preliminary inspection of the results suggested that masculine reactions increased as testing progressed. It appeared possible that our findings might be strongly affected by the techniques of the present study. These techniques involved repeated presentation

TABLE 2. FREQUENCY OF EACH TYPE OF MASCULINE RESPONSE SHOWN BY 15 FEMALE RATS AT VARIOUS STAGES IN THE TEST SERIES

	Average Number of Responses Per Test		
	Sexual clasp	Palpation and pelvic thrusts	Complete pattern
First 199 tests	2.3	.7	.05
Last 191 tests	3.4	1.3	.03
First 5 tests	1.9	.4	0
Remaining tests (M=20.8 per rat)	3.6	1.1	.04

of 'receptive stimulus' females to the experimental females, the occurrence of occasional pseudopregnancies of experimental animals caused by taking vaginal smears, and by copulation with vasectomized males, and perhaps still other undetected or seemingly extraneous factors. If such influences raised the frequency of masculine responses one might logically expect an increase in male behavior in successive tests.

Comparisons between results of tests conducted during the first and second halves of the experiment revealed some evidence of increased masculine activity during later tests. In the first 199 tests 15 females displayed masculine reactions a total of 701 times; whereas the same behavior appeared 890 times during the last 191 tests of these animals. Somewhat greater differences appear if we compare the average performance during the first 5 tests with that shown in all subsequent tests. Data presented in table 2 illustrate these points.

Group records for the first 17 tests which are shown in figure 2 reveal that as the experiment progressed there was a tendency for more and more females to show masculine mating responses. The first appearance of male behavior occurred in the first test for 8 females, in the second test for 4 females, in the third test for one female and in the fourth test for one female. In figure 2 there is also some evidence of a gradual increase in the number of responses shown by the same female in successive tests (average responses per positive female).

Further indication that continued testing increases the female rat's tendency to show masculine mating reactions is found in data presented in table 3. The sexual clasp occurred very early in the testing series for all 15 animals. Palpation with pelvic thrusts appeared somewhat later, after approximately 5 tests. The complete pattern, which was the last response to appear, usually did not occur before the twelfth test.

Repeated testing apparently resulted in some increase in the masculine behavior shown by our females, but the magnitude of the increase is relatively slight, and we feel that the reactions under examination cannot be regarded as artifacts produced by the experimental methods.

Feminine mating responses in experimental females. Although casual observation indicated that the females used in the present work displayed normal feminine receptivity when in heat, this point was checked quantitatively. Data presented in table

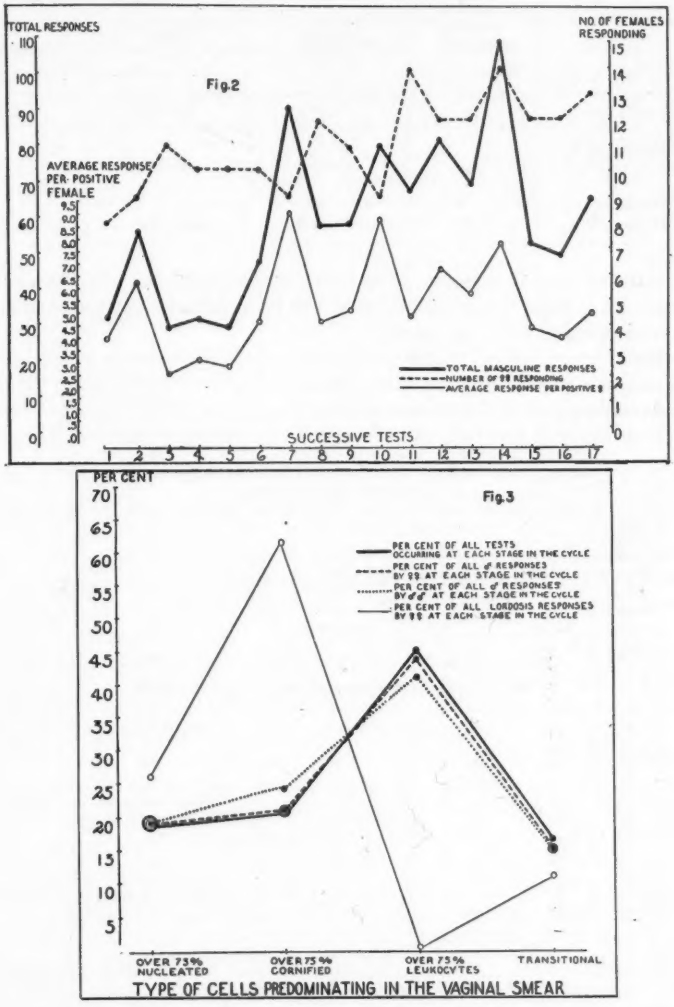

TOTAL RESPONSES
NO. OF FEMALES RESPONDING
Fig.2
AVERAGE RESPONSE PER POSITIVE FEMALE
TOTAL MASCULINE RESPONSES
NUMBER OF ♀♀ RESPONDING
AVERAGE RESPONSE PER POSITIVE ♀
SUCCESSIVE TESTS
PER CENT
Fig.3
PER CENT OF ALL TESTS OCCURRING AT EACH STAGE IN THE CYCLE
PER CENT OF ALL ♂ RESPONSES BY ♀♀ AT EACH STAGE IN THE CYCLE
PER CENT OF ALL ♂ RESPONSES BY ♂♂ AT EACH STAGE IN THE CYCLE
PER CENT OF ALL LORDOSIS RESPONSES BY ♀♀ AT EACH STAGE IN THE CYCLE
OVER 75% NUCLEATED
OVER 75% CORNIFIED
OVER 75% LEUKOCYTES
TRANSITIONAL
TYPE OF CELLS PREDOMINATING IN THE VAGINAL SMEAR

Fig. 2. Masculine responses shown by 15 females in 17 successive tests.
Fig. 3. Distribution of tests, male's copulatory behavior, females' masculine responses and females' receptive behavior over the 4 stages of the vaginal estrous cycle.

4 indicate that 11 of the 15 females were most receptive when the vaginal smear contained a preponderance of cornified epithelium. Four of these 11 females were also receptive at other stages in the cycle. One animal was most receptive when the vaginal smear showed chiefly nucleated epithelium; another rat was most receptive in

TABLE 3. POINT IN THE TEST SERIES AT WHICH EACH TYPE OF MASCULINE RESPONSE FIRST APPEARED

Response	Number of Females Showing Response	Number of Test in Which Response First Appeared		
		Earliest instance	Latest instance	Average
Sexual clasp	15	1st test	4th test	1.7
Palpation and pelvic thrusts	14	1st test	6th test	5.5
Complete pattern	5	6th test	24th test	12.6

the transitional stage (late nucleated or early cornified epithelial cells). With the exception of *rat 45* these 13 animals exhibited heat behavior as readily and as frequently as the stock breeders from our colony.

It should be emphasized that no attempt was made to determine maximum receptivity, and tests were not spaced so as to occur at any particular stage in the estrous cycle. Accordingly, it is safe to assume that the data presented represent less than the highest degree of heat behavior of which the females were capable. The chances

TABLE 4. RECEPTIVE BEHAVIOR OF 15 FEMALES WHICH WERE ALSO OBSERVED FOR MASCULINE RESPONSES

Rat No.	Total Tests During Most Receptive Stage	Times Female Mounted by Male	Times Female Showed Lordosis	% of Mounts Eliciting Lordosis	Total Ear-wiggle Responses	Total Hopping Responses	Notes
Most receptive during cornified stage of vaginal estrus							
9	6	34	11	32	6	6	Also receptive in nucleated stage
31	6	94	16	17	0	1	
34	5	42	22	52	17	17	
35	7	92	72	78	54	38	
36	2	17	8	47	6	7	Also receptive in nucleated and transitional stage.
37	7	69	31	45	49	33	
38	8	36	12	33	7	2	
39	6	55	19	35	6	6	
42	4	35	27	77	19	14	Also receptive in transitional stage
43	8	85	30	35	26	10	Also receptive in nucleated stage
45	6	46	1	2	5	0	Receptivity dubious
Most receptive during nucleated stage of vaginal estrus							
3	11	89	56	63	104	88	
Most receptive during transitional stage of vaginal estrus							
33	5	54	22	40	24	11	Also receptive in nucleated and transitional stages
Never receptive							
5	28	148	0	0	0	0	
44	13	76	0	0	0	0	

of catching any single animal just at the peak of the receptive period were slight. It is possible therefore that *rats 5* and *44* would have exhibited receptive behavior in a testing series designed to measure responsiveness at every point in the cycle.

The relationship between vaginal smears and receptive behavior in those females which accepted the male can be illustrated roughly by two comparisons. During either the nucleated, transitional or cornified stages of vaginal estrous the 13 animals the receptive behavior of which is shown in table 4 received a total of 81 tests (range 2–11). In the course of these tests the females were mounted by males 748 times (range 17–94), and showed lordosis 327 times (range 1–72). Approximately 44 per cent of the instances of mounting by the males elicited lordosis. In the same tests the

TABLE 5. FREQUENCY OF RECEPTIVE BEHAVIOR SHOWN BY 13 FEMALES AT EACH STAGE OF VAGINAL ESTROUS CYCLE

	Type of Cells Predominating in Vaginal Smear			
	Over 75% nucleated	Over 75% cornified	Transitional	Over 75% leukocytes
Percentage of 345 tests occurring at each stage of the cycle	19	21	15	45
Percentage of 2762 mounts by males occurring at each stage of the cycle	19	24	15	42
Percentage of 415 lordosis responses by females occurring at each stage of the cycle	26	63	11	0

females exhibited the ear-wiggling reaction 323 times (range 0–104), and the hopping response 233 times (range 0–88). These same females received a total of 155 tests (range 7–24) during periods when the vaginal smears showed over 75 per cent leukocytes. In the course of these tests the females were mounted by vigorous males a total of 1,148 times (range 22–221), but none of the animals displayed lordosis, ear-wiggling, or hopping reactions.

Degrees of receptivity exhibited at different stages in vaginal estrus are further revealed by data presented in table 5. The important features of these data are, *a*), the close parallel between distribution of tests and distribution of the attempts of the male rats to copulate with the experimental female, and *b*), the lack of relationship between the foregoing variables and the distribution of the execution by the females of the receptive response (lordosis). For example, 21 per cent of the total number of tests occurred during the cornified stage, and 24 per cent of the males' attempts to copulate took place during this same period; but 63 per cent of the 415 lordosis responses of the females were concentrated in this stage of the cycle. Conversely, 45 per cent of the 345 tests occurred during diestrus (over 75% leukocytes), and 42 per cent of the 2,762 copulatory attempts of the males appeared in the same tests; but not a single lordosis response was elicited from any female during these tests. Data presented in table 5 merely repeat what has long been known, that the female rat's display of receptive behavior is closely correlated with vaginal estrus; but they are shown here as a basis for comparisons with the masculine copulatory behavior of the female to be discussed below.

Relation of masculine behavior to estrus. In earlier reports (25, 26) the senior author described masculine copulatory reactions shown by both virgin and sexually experienced female rats. In all instances, the females in question were in estrus, and displayed normal feminine receptivity when approached by the male.

Using the results of the present experiment, an attempt was made to compare the frequency of masculine sexual responses, shown during tests in which the experi-

mental females were receptive, with the frequency of the same responses during tests in which there was no indication of feminine receptivity. A female was scored as 'receptive' if she showed lordosis in response to at least one third of the male's mounts (i.e., if 33 per cent of the male's mounts elicited lordosis). Nonreceptivity was indicated by refusal to exhibit lordosis. Tests in which receptive behavior occurred once or twice were not counted in either category. The records of 3 females that never showed a high degree of receptivity were excluded from these calculations. Data presented in table 6 refer to behavior occurring during the first and final period of the

TABLE 6. COMPARISON OF MASCULINE COPULATORY RESPONSES SHOWN BY 12 FEMALES DURING TESTS IN WHICH THEY WERE HIGHLY RECEPTIVE AND TESTS IN WHICH THEY WERE TOTALLY NON-RECEPTIVE

Condition of Female	Total Tests in Each Condition	Sum of Masculine Responses	Average Masculine Response per Test
Very receptive	40	78	1.95
Non-receptive	265	265	3.35

test, at which times the experimental female was in the cage with a 'receptive stimulus' female and the male was not present (see description of experimental methods).

Our findings as shown in table 6 reveal a tendency for more frequent appearance of masculine reactions during tests in which females were not sexually receptive. The critical ratio $(m_1-m_2/\sqrt{\sigma_1^2+\sigma_2^2})$ of the difference between the average masculine response per test under the two conditions was 2.96, which suggests the existence of a true difference. Detailed analysis showed that this tendency applied equally to all 3 types of masculine response studied.

In view of the negative relationship between receptivity and frequency of masculine copulatory reactions, it was deemed advisable to attempt to correlate the appearance of masculine behavior with the types of cells present in the vaginal smear. The results of this analysis are presented in tables 7 and 8. Both of these tables show conclusively that the masculine mating reactions of the experimental females were quite evenly distributed throughout all stages of the vaginal estrous cycle.

As may be seen in table 7, male behavior appeared in approximately the same proportion of tests (73 to 80 per cent) regardless of the stage of vaginal estrus during which those tests were given. Seventy-four tests occurred when smears contained over 75 per cent of nucleated epithelial cells, and 177 tests were given during periods when smears showed over 75 per cent leukocytes. Masculine copulatory reactions were exhibited in 73 per cent of the first and 72 per cent of the second group of tests. Analyzing the relationships in another way, it is shown in table 8 that the distribution of masculine responses among the various stages of vaginal estrus follows almost exactly the distribution of tests given over the same stages.

The difference between male and female copulatory responses shown by the 15 females at each point in the estrous cycle is further illustrated in figure 3. The distribution of tests (heavy line) is followed closely by the distribution of the masculine reactions of the females (broken line), and by the distribution of copulatory attempts on the part of the stimulus males (dotted line). This is to say that masculine mating responses by both experimental females and stimulus males vary directly with the frequency of tests, and that this relationship does not fluctuate with changes in the vaginal smear picture.[2] In contrast, the distribution of receptive behavior on the part

[2] The rise in the curve of test frequency at the point of diestrus is occasioned by the temporal span of the various stages of the complete cycle. Since leukocytes predominate for about half of the 4-day cycle the fact that 45 per cent of all tests occurred during this stage is easy to understand.

of the experimental females is closely related to the type of smear, and is relatively independent of either the frequency of the tests or copulatory attempts executed by stimulus males (light line).

Behavior of ovariectomized adult females. The apparent lack of relationship between the vaginal estrous cycle and the appearance or frequency of masculine copulatory performance by the experimental females led us to believe that this behavior might be independent of ovarian hormones. As an initial test of this hypothesis 5 of the females were ovariectomized, set aside for from 21 to 43 days, and then re-tested for male sexual behavior. Prior to castration the 5 animals were given a total of 121

TABLE 7. PERCENTAGE OF TESTS IN WHICH MASCULINE RESPONSES OCCURRED AT EACH STAGE OF THE VAGINAL CYCLE, 15 FEMALES

	Type of Cells Predominating in the Vaginal Smear			
	Over 75% nucleated	Over 75% cornified	Over 75% leukocytes	Transitional
Total number of tests occurring in each stage	74	83	177	56
Per cent of tests in which masculine behavior appeared	73	76	72	80

TABLE 8. RELATIVE PROPORTIONS OF ALL TESTS AND OF ALL MASCULINE RESPONSES OCCURRING AT EACH STAGE OF THE VAGINAL CYCLE, 15 FEMALES

	Type of Cells Predominating in the Vaginal Smear			
	Over 75% nucleated	Over 75% cornified	Over 75% leukocytes	Transitional
Percentage of the total of 390 tests occurring in each stage of vaginal estrus	19	21	45	15
Percentage of the total of 1,608 masculine responses occurring in each stage of vaginal estrus	19	21	44	16

tests, in 84 (69 per cent) of which masculine reactions occurred. After removal of the ovaries the females received 10 tests, and male behavior occurred in all but one test (90%). The average number of masculine responses per test before ovariectomy was 4.6 per test (390 responses in 84 positive tests). After castration there occurred 458 responses in 92 tests, or an average of 5 per positive test.

To re-check the tentative conclusion that there is no relationship between vaginal estrus and the appearance of masculine mating reactions, each of the 5 ovariectomized females was brought into estrus twice. An injection of 500 R.U. of estradiol benzoate[3] was followed 48 hours later by injection of .5 mg. of progesterone.[3] These heavy dosages produced complete cornification for several days and all females became sexually receptive, although not intensely so.

Injection of estrogen and progesterone and consequent induction of estrus had no pronounced effect upon the tendency of the castrated females to show masculine copulatory reactions. The percentage of tests in which male behavior occurred was somewhat higher after castration than before. Prior to ovariectomy masculine re-

[3] We are indebted to the Schering Corp., Bloomfield, N. J. for the estradiol benzoate (Progynon-B) employed, and to Ciba Pharmaceutical Products, Inc., Summit, N. J. for the progesterone (Lutocylin) used in the present work.

sponses were shown in 69 per cent of 121 tests. Following operation and the injection of ovarian hormones 81 per cent of 113 tests revealed the execution of such responses. However, this post-castration increase in percentage of positive tests cannot be attributed to the injected hormones for it was apparent in 10 tests given after operation but before hormone treatment (9 of 10 tests positive).

The increase in masculine sexual behavior was independent of variations in the vaginal condition. In figure 4 the percentage of all tests occurring at each stage in the natural and induced vaginal cycles is represented by solid lines, and the percentage of all masculine responses shown during these same stages is shown by broken lines. In both the natural and artificially created vaginal estrus the distribution of masculine responses closely follows that of tests, indicating an absence of correlation between cycle stages and the tendency to exhibit masculine behavior.[4]

It is known that repeated testing with receptive females presented under constant conditions has a marked effect upon the sexual responsiveness of males of many species (27). A male accustomed to receiving receptive females in a certain cage may attempt to mate with any animal of proper size that is offered in that particular cage. The castrated females described above had received 'receptive stimulus' females in the test cage during many preoperative tests, and it appeared possible that the resultant 'conditioning' might have contributed to the survival of masculine performance after loss of the ovaries. To test this possibility 5 virgin females from the colony were ovariectomized and examined for masculine copulatory behavior 30 days after operation.

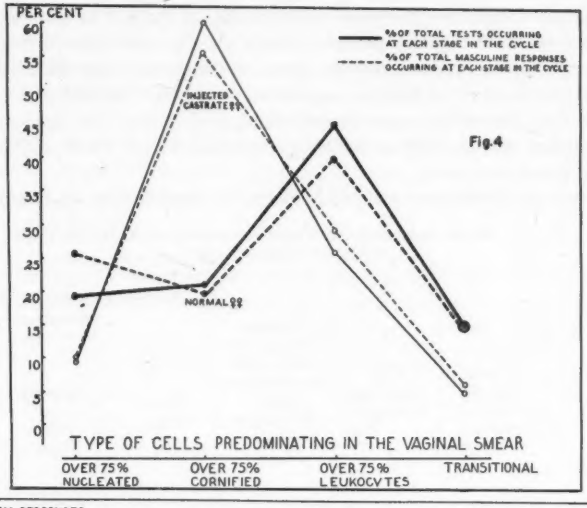
In 10 tests, 4 of the 5 castrates exhibited a total of 134 masculine copulatory reactions (95 clasp, 38 palpate and thrust, 1 complete pattern). The proportion of the small group showing this behavior, and the frequency with which each type of response was executed demonstrated that females castrated in adulthood but without the experience of repeated sex tests were comparable to experienced castrates in the tendency to exhibit elements of the masculine mating pattern.

In the series of 10 tests, the male behavior of these 4 castrated females showed some tendency to increase with experience. The total number of masculine responses in each succeeding test were as follows: 2, 0, 1, 6, 18, 30, 14, 12, 19, 32. The sexual clasp was shown by the 4 animals after an average of 3.5 tests; palpation with pelvic thrusts first occurred after an average of 5.2 tests; and the complete pattern appeared in the 9th test. Repeated testing seemed to affect the masculine behavior of castrated females in much the same fashion as it did that of intact animals.

Behavior of prepubertally-ovariectomized females. Before concluding that the ovaries are not essential to the execution of masculine mating behavior by female rats we considered the possibility that ovarian hormones secreted after puberty but prior to castration might exert some effect permitting the occurrence of male behavior in the adult female after ovariectomy. There was a hypothetical possibility that the nervous system is permanently 'sensitized' (in Steinachian terminology) by postpubertal ovarian hormones; and that once such sensitization is achieved the hormones responsible are no longer needed.

To test this hypothesis, 17 prepubertal female rats were ovariectomized at 21 to 27 days of age and later observed for male behavior. These animals were raised together in one large cage from 21 days of age, and thus from the day of weaning had no contact with males or intact females. Beginning approximately 3 weeks after castration each of the operated females was given a series of fifteen 10-minute sex tests with a cage mate (also prepubertally ovariectomized). When they were 61 to 67 days old the

[4] The shift toward a majority of tests during cornified stage of induced estrus simply reflects the prolonged cornification resulting from very large doses of estrogen.


PER CENT
% OF TOTAL TESTS OCCURRING AT EACH STAGE IN THE CYCLE
% OF TOTAL MASCULINE RESPONSES OCCURRING AT EACH STAGE IN THE CYCLE
INJECTED CASTRATES ♀♀
NORMAL ♀♀
Fig. 4
TYPE OF CELLS PREDOMINATING IN THE VAGINAL SMEAR
OVER 75% NUCLEATED
OVER 75% CORNIFIED
OVER 75% LEUKOCYTES
TRANSITIONAL

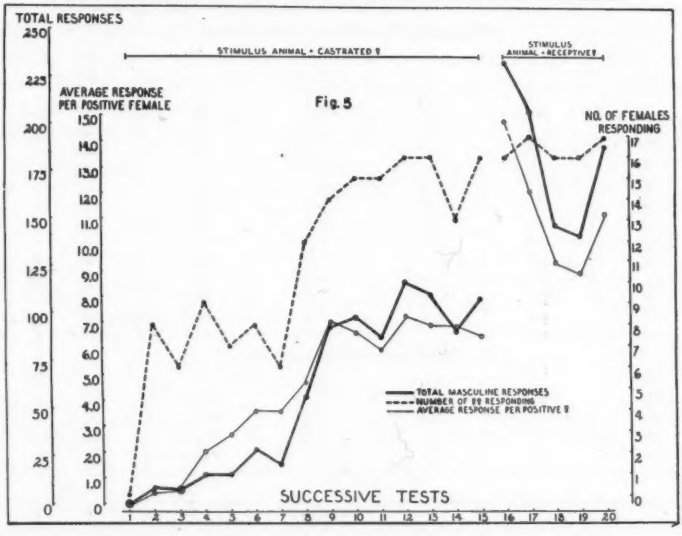


Fig. 4. Distribution of tests and masculine responses over the 4 stages of the vaginal estrous cycle for 5 females tested as normals and then as injected castrates.
Fig. 5. Masculine responses shown by 17 prepubertally castrated females in 20 successive tests.

castrates were given five 10-minute tests with a receptive female. Males were never used in this test series.

The results of tests with ovariectomized cage-mates and with receptive females are shown in table 9. Masculine copulatory reactions were observed 1,787 times. Of this total, 1,414 responses consisted of the sexual clasp, 361 palpation with pelvic thrusts, and 14 the complete pattern. Test-to-test records for the group as presented in table 9 are illustrated graphically in figure 5.

Three main trends are observable in the results of the first 15 tests, during which young castrates were tested together in pairs. As the experiment progressed there was an increase in the proportion of the group which showed masculine behavior. The increase in the number of females responding positively resulted in a concomitant rise in the total masculine responses occurring in each test. At the same time the average number of masculine responses per positive female showed a fairly steady increase in successive tests.

These various trends are susceptible to several explanations. *a*). Results obtained

TABLE 9. MASCULINE COPULATORY RESPONSES SHOWN BY 17 PREPUBERTALLY OVARIECTOMIZED FEMALES

Successive Tests	Number of Females Showing Masculine Responses	Total Masculine Responses	Average Number of Masculine Responses per Positive Female per Positive Test	Total of Each Type of Masculine Response		
				Sexual Clasp	Palpation and pelvic Thrusts	Complete Pattern
with castrated female as stimulus animal						
1	0	0	0	0	0	0
2	8	11	1.4	11	0	0
3	6	9	1.5	9	0	0
4	9	18	2.0	17	1	0
5	7	18	2.6	17	1	0
6	8	28	3.5	24	4	0
7	6	21	3.5	21	0	0
8	12	56	4.6	55	1	0
9	14	96	6.9	81	15	0
10	15	99	6.6	87	12	0
11	15	88	5.9	84	4	0
12	16	117	7.3	103	14	0
13	16	110	6.9	97	13	0
14	13	89	6.8	78	11	0
15	16	104	6.5	94	10	0
with receptive female as stimulus animal						
16	16	235	14.7	158	74	3
17	17	206	12.1	145	54	7
18	16	148	9.3	101	46	1
19	16	143	8.9	103	39	1
20	17	191	11.2	129	62	2

in successive tests with intact or castrated adult females revealed a tendency toward an increase in the number of females responding and in the average number of responses per positive female per test. It is permissible therefore to regard experience in the testing situation as one factor contributing to the progressive increase in responsiveness shown by prepubertal castrates.

b). All 17 of the young animals were tested on the same days, and since there was a 6-day variation in date of birth, the females were different ages at the time of the first test. That these differences in age may have influenced the results is revealed by data presented in table 10. The oldest females tended to show masculine responses earlier in the test series than did younger rats. The second and third columns in table 10 suggest an inverse relationship between age at the time of the first test and the number of the test in which masculine behavior first appeared. The fourth column indicates that such behavior usually first occurred at 43 to 45 days of age. Therefore

part of the increase in masculine responses shown in table 9 and figure 4 may be interpreted as reflecting this function of the increasing age of the test animals.[5]

In *tests 16* to *20* the prepubertal castrates were given receptive females as stimulus animals. Table 9 and figure 5 show that this change in experimental conditions resulted in an abrupt and marked increase in masculine mating responses shown by the experimental females. In tests with receptive females the prepubertal castrates showed an increase in the number of sexual clasps, and in palpation with pelvic thrusts; and the complete pattern, which had not been elicited in any of the preceding tests, appeared during this period.

TABLE 10. AGE OF CASTRATED FEMALES AT TEST IN WHICH MASCULINE RESPONSES FIRST APPEARED, 17 FEMALES

Number of Cases	Days of Age at First Test	Average Number of the Test in Which First Masculine Response Appeared	Average Days of Age at Test in Which First Masculine Response Appeared
2	43	3.0	45.5
8	40	4.4	44.1
4	39	4.3	43.0
3	37	6.7	44.0

Table 11 presents comparisons between the masculine copulatory reactions shown in response to a receptive female by intact adult females, adults castrated before experience in sex tests, and prepubertal castrates. Also included in this table are the records of the prepubertal castrates in tests when the stimulus animal was a second prepubertally castrated female. Variability in the size of the groups, and in the

TABLE 11. COMPARISONS OF MASCULINE COPULATORY BEHAVIOR SHOWN BY INTACT ADULT FEMALES, CASTRATED ADULT FEMALES AND PREPUBERTALLY CASTRATED FEMALE RATS

Group	Stimulus Animal	Number of Tests per Female	Sexual Clasp			Palpation and Pelvic Thrusts			Complete Pattern		
			% of group responding	% of tests in which positive cases showed the response	Average frequency of response per positive test	% of group responding	% of tests in which positive cases showed the response	Average frequency of response per positive test	% of group responding	% of tests in which positive cases showed the response	Average frequency of response per positive test
15 intact adults	receptive female	20	100	76	4.18	93	34	2.96	27[1]	5	1.57
5 adult castrates	receptive female	10	80	55	4.32	80	28	3.45	20	10	1.00
17 prepubertal castrates	receptive female	5	100	95	7.63	77	79	4.83	24	40	1.30
17 prepubertal castrates	prepubertally castrated female	15	100	92	4.51	53	32	2.14	0	0	0

[1] Subsequent tests raised this figure to 33.

number of tests given undoubtedly contributes to some of the intra-group differences in responsiveness to the receptive female. Differences which seem large enough to be significant can be traced to variation in the ages of the experimental groups.

[5] This evidence should not be taken to mean that masculine copulatory behavior cannot be elicited in female rats prior to 43 days of age. On the contrary, experiments now in progress reveal the appearance of such reactions at a much earlier age. Present results merely suggest that for the particular females investigated, and under the conditions imposed, the responses involved tended to appear at approximately 44 days of age.

All females with the exception of one adult castrate showed the sexual clasp; but juvenile castrates showed this response in a much higher proportion of the tests than did adult females. This difference cannot be due to ovariectomy for the adult castrates were even less responsive than intact rats of comparable age. During those tests in which the sexual clasp occurred it was executed nearly twice as frequently by the prepubertal castrates as by females in the other two groups. Again, the variation seems traceable to an age factor rather than absence of ovaries since the record of adult castrates approximates that of intact adults. In comparison with normal adults, a somewhat lower percentage of the young castrates showed the palpate and thrust response, but those juvenile females which did exhibit this reaction executed it in a much higher proportion of the tests and a greater number of times per positive test than did the adult females.

Approximately the same proportions of the 3 groups showed the complete pattern, and the frequency of its execution during tests in which it did appear was about the same for all groups. However those prepubertal castrates which displayed the complete pattern tended to do so in 40 per cent of the tests, whereas adult animals responded with this reaction in only 5 to 10 per cent of all tests.

Comparison of the reactions of prepubertal castrates to another castrate and to a receptive female reveals that the latter was uniformly a more effective stimulus animal. When a receptive female was presented, each type of masculine copulatory response occurred in a higher percentage of tests and was exhibited more frequently during the positive tests.

DISCUSSION

The findings presented above are at variance with earlier reports in several respects. Other experimenters have regarded the execution of masculine copulatory reactions by female rats as very rare, whereas 18 of the 20 intact adult females which we have described showed such behavior. This apparent contradiction is undoubtedly due in part to the fact that nearly all of the previous reports have been based upon casual observation under uncontrolled conditions. The behavior in question probably appears much more frequently when 2 females are repeatedly tested together than when a number of females, or males and females are kept together in a large cage and observed incidentally.

We have interpreted as masculine behavior the execution of single elements which make up the copulating male's total response. Other investigators may have observed female rats exhibiting the sexual clasp, or palpation with pelvic thrusts, but preferred to restrict the term 'male sex behavior' to the appearance of the reaction which we have designated as the 'complete pattern.'

A part of the frequency with which females herein reported exhibited masculine responses has been shown to be the result of repeated testing.

There is a possibility that females from our particular colony are more active in a masculine direction than are females from other stock. This aspect of the problem has not yet been checked.

The apparent lack of correlation between ovarian cycles and the appearance of masculine sexual behavior presents several problems. Previously one of us (25, 26), in describing this behavior tended to regard it as 'abnormal behavior' associated with estrus. This misconception arose because we observed masculine behavior only in receptive females. However, nonreceptive females were rarely given opportunity to react at all. The masculine behavior was seen in females used as stimulus objects in an experiment upon copulatory behavior of the male rat. Females were put with

males at the start of a day's testing. Nonreceptive females were returned to the colony, while receptive animals were placed together in one cage where they were kept when not in use with males. Under such conditions receptive females in the resting cage were seen to mount each other. From such observation it was incorrectly inferred that this behavior was associated with receptivity and therefore with estrus. We had no proof that nonreceptive females would fail to respond similarly, and present results show that they do.

It seems not unlikely that reports of a close relationship between heat and male-like mounting behavior in the female of various mammalian species may reflect similar absence of systematic observation of diestrous females. In fact, there is little indication that any investigator has carefully and repeatedly tested for the appearance of this behavior in diestrous females confronted with a receptive female as the stimulus animal. In general, emphasis has been placed upon masculine responses seen at the time of heat, and evidence for the absence of such responses at other periods is either scanty or totally lacking. It follows, therefore, that the generally accepted belief that masculine mounting behavior is associated with heat, and thus, by inference, dependent upon ovarian hormones, may be the result of purely fortuitous factors.

One must except from the foregoing generalization the studies of Ball (23) and those of Young and his co-workers (5, 28, 29, 30). Ball investigated the effects of testosterone on sexual behavior in the female rat and concluded that untreated females rarely exhibit the male pattern. Ball did note that the normal receptive females (used as stimulus animals) mounted the testosterone-treated females, but interpreted this behavior as due to some particularly stimulating aspect of the behavior of the testosterone-treated animals. Furthermore, Ball reports one instance in which a normal female in diestrus mounted a receptive rat. Thus differences between present findings with intact adult females and those reported by Ball consist merely of lack of agreement as to the frequency of masculine mating reactions in the female. The two studies agree in finding that the responses are present, and that they may be exhibited both during estrus and diestrus.

Observations on female guinea pigs are more difficult to reconcile with present results unless one dismisses the problem by categorizing it as species difference. The female guinea pig's display of mounting behavior is temporally related to receptivity, and Young and Rundlett (28) have suggested the possibility that mounting, like the heat response, is stimulated by the synergistic action of estrogen and progesterone. This hypothesis cannot be applied to our findings with the rat in view of the appearance of masculine responses in adult and prepubertal castrates. It should be noted, however, that, in the guinea pig, heat and mounting activity occasionally occur independently, and when they do appear simultaneously there is no relation between frequency of mounting and length of the heat period (5).

We have no positive evidence to indicate the hormonal basis of masculine mating reactions in the female rat. Obviously ovarian secretions are not essential. If it is assumed that the appearance of male behavior is always dependent upon androgens (a wholly gratuitous assumption) it is possible to speculate on the source of the androgen involved in this instance. The adrenal cortex might be regarded as a possible source of androgens, but speculation on this point seems fruitless at the present stage of knowledge. Certainly there is no reason to associate the behavior under consideration with any organic dysfunction (e.g. cortical adrenal tumor). More probably the display of masculine copulatory responses is to be regarded as typical of female rats from our colony when confronted with a particular stimulus situation.

The negative relationship between receptivity and maculine activity cannot be explained yet to our satisfaction. At first glance there seems to be direct contradiction involved in the finding that receptivity is positively related to vaginal estrus and negatively related to the appearance of male behavior, while at the same time there is no relation between masculine behavior and vaginal estrus. In an attempt to resolve this apparent conflict we suggest that the positive relationship between vaginal estrus and receptivity is far from a perfect correlation; while the negative relationship between receptivity and male behavior, although showing a statistically significant difference, is not extreme.

From the point of view of psychobiology, it is interesting to note the similarities between the masculine sexual behavior of male and female rats. There are at least two points for comparison. If a group of either males or females is repeatedly tested with receptive females, the proportion of the group exhibiting masculine behavior increases during the first few tests and then remains fairly constant. In groups of either sex there are some animals which are either more easily aroused sexually, or less easily inhibited by the testing situation, and therefore copulate or attempt to copulate with the receptive female in the first tests. A second class of rats fails to show masculine behavior in initial tests but does so later in the test series. A final group, both among males and females, never attempts to mount the receptive female.

A second comparison of the masculine sexual reactions shown by male and female rats lies in the definition of the adequate stimulus pattern. It has been shown that male rats exhibit copulatory behavior most readily and frequently in response to a receptive female. The phenomena of sexual excitability intimately involve sensitivity to a pattern of multisensory stimulation; and in the rat this responsiveness to a particular stimulus pattern appears to be mediated by an inherited nervous organization (27, 31, 32). We have described above an immediate increase in masculine behavior shown by prepubertally castrated females when receptive females were substituted for castrates as the stimulus animal. We interpret this increase as a reflection of heightened sexual excitement on the part of the experimental females. In earlier papers (23, 25, 26) it has been suggested that female rats inherit a neuromuscular organization capable of mediating the copulatory pattern characteristic of the male of their species. Present findings indicate that in the female as in the male this motor pattern is elicited most readily by that stimulus pattern presented by the receptive female.

It is always taken for granted that in many types of instinctive behavior the same stimulus→response relationships exist for both sexes (e.g. hunger→food→eating, or, pain→avoidance reactions). Usually, however, the mating patterns of the sexes have been regarded as sex-specific. It is therefore of definite theoretical interest to observe that when the masculine copulatory response appears, it is linked with a particular stimulus pattern; and that this relationship of stimulus to response is constant regardless of the sex of the reacting animal. For some purposes the reactions usually regarded as masculine copulatory behavior might better be defined in terms of a stimulus→response relationship with no reference to the sex of the animal showing the behavior.

SUMMARY

Twenty adult female rats were tested for the appearance of both feminine and masculine copulatory reactions. Eighteen animals showed sexual receptivity when mounted by males during estrus. Eighteen rats responded to receptive females with the execution of certain elements of the masculine copulatory pattern. Three types

or grades of mating behavior characteristic of the copulating male and shown by the females herein described are the sexual clasp, clasping with palpation and pelvic thrusts, and the 'complete pattern' which includes all of the foregoing plus dismounting after a final, vigorous pelvic thrust and frequently involves licking of the genitals.

The feminine sexual pattern (receptivity) occurred during the period of vaginal estrus and could never be elicited when the smear consisted chiefly of leukocytes. Masculine copulatory reactions were performed with equal frequency at all stages of the estrous cycle. Castration of 5 females eliminated receptive behavior but had no obvious effect upon the execution of the male pattern. Injection of estrogen and progesterone revived receptivity and did not alter masculine behavior.

Seventeen female rats were ovariectomized at 21 to 27 days of age and tested between the ages of 39 and 81 days. All of these juvenile castrates showed the sexual clasp. Thirteen animals clasped with palpation and pelvice thrusts, and 4 females exhibited the complete pattern. When tested with other female castrates and then with a receptive female the prepubertal castrates showed a greater amount of masculine behavior in response to the latter stimulus animal.

It is concluded that the majority of female rats from the colony studied possess a neuromotor organization capable of mediating the masculine copulatory pattern. It is further concluded that in the female this motor pattern appears most readily in response to the same stimulus pattern by which it is evoked in the male, namely a multi-sensory pattern of stimulation presented by the receptive female. Finally, it is concluded that the masculine sexual reactions of the female rat are not dependent upon ovarian hormones.

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EFFECTS OF ENDOCRINES ON AGE CHANGES IN THE EPIPHYSEAL AND ARTICULAR CARTILAGES[1,2,3]

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THIS IS a report of the growth and age changes in the skeletal tissues of mice, rats and guinea pigs.

Normal time curve of the skeletal tissues. During endochondral ossification new cartilage is formed from a germinal layer of cartilage. The cartilage cells assume columnar arrangement and, at a certain phase of their development, they undergo hypertrophy. The hypertrophic cells calcify, are destroyed by constituents of the bone marrow and are replaced by primary osseous-spicules. This primary bone is subsequently resorbed by bone marrow. As long as these processes proceed at an even rate, the cellular pattern of the epiphyseal disks is regular, and the growth zones remain open. A decrease of growth or an increase of regression and resorption of the cartilage lead to a narrowing and progressive closure of the epiphyseal disks; on the other hand, an increase of proliferative or a decrease of regressive or resorptive processes may cause an enlargement of the growth zones. Increased regression and resorption of cartilage accelerate perforations of the epiphyseal disks; decreased resorption causes a retardation of the perforations of the epiphyseal zones and a persistence of the primary bony trabeculae.

During the life span of the mouse, three main phases of skeletal development and aging can be distinguished (1). During the first phase, growth of cartilage predominates, but towards the end of this phase the growth of the cartilage decreases, and the growth zones begin to narrow. During the second phase, regression of cartilage and formation of bone are prominent. The epiphyseal zones continue to narrow; they become inactive and ossify. During the third phase, regression of such cartilage as is left proceeds, but resorption of cartilage and bone is the main feature. The epiphyseal growth zones are thinned out and are perforated by constituents of the bone marrow. The epiphyseal and diaphyseal cavities unite, although to a varying degree. But, under normal conditions, complete epiphyseo-diaphyseal union does not occur in the mouse. In old age, resorption of bone surpasses the formation of bone. While these phases are quite definite, to some extent they may overlap.

Skeletal development proceeds at a faster rate in females than in males, in breeding females more rapidly than in virgins, in strains D, C_{3H} and A, more rapidly than in strains C57, CBA, New Buffalo and Old Buffalo. In mice of strain C57, the first phase, the period of growth of cartilage, lasted up to the 4th month of life, when regressive

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[2] The experiments referred to in this paper were conducted in the Laboratory of Research Pathology, Washington University, School of Medicine, Saint Louis, Missouri.

[3] The investigations were carried out with the aid of grants from the Committee in Research in Endocrinology of the National Research Council, from the Jane Coffin Childs Memorial Fund for Medical Research, and from the International Cancer Research Foundation given to Dr. Leo Loeb, from the Committee on Scientific Research of the American Medical Association, and from the Albion O. Bernstein fellowship in Pathology, New York University, College of Medicine.

changes set in. In mice of strain D, a corresponding condition already was noted at the age of 8 weeks (fig. 1). In strain C57, perforations in the epiphyseal disk appeared first towards the end of the first year of life. In strain D, such changes were observed as early as at the age of 6 months, and at 7 months, the extent of these perforations surpassed that seen in strain C57 at 14 months. Moreover, strains C_{3H}, D, and A showed a tendency to deposit more bone than strains C57 and CBA (fig. 2, 3, 4).

Aging processes in the joints. With advancing age, the cartilage of the joints may

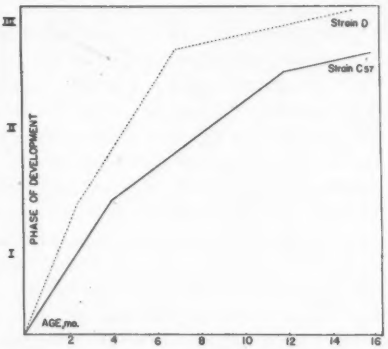

Fig. 1. TIME CURVES OF SKELETAL DEVELOPMENT and aging of mice of strains C57 and D. I. Phase of predominance of proliferation of cartilage. II. Phase of predominance of regression of cartilage. III. Phase of predominance of resorption of cartilage and bone.

undergo changes of varying degree. The following gradation of these articular changes was made: *Grade I* is characterized by slight hyperplasia and hypertrophy of the articular cartilage; *Grade II*, by a moderate degree of both growth and regression of cartilage; and *Grade III*, by marked proliferation, hypertrophy and regression of cartilage (fig. 5, 6). These lesions were comparable to those of degenerative joint disease or osteoarthritis in man. The incidence and severity of articular changes at a given age were greater in strains with a faster rate of skeletal aging than in those with a slower rate.

Hence the skeleton has its own time curve. This finding is in agreement with the conclusion of Loeb (2), that each tissue and organ has its own characteristic time

TABLE 1. INCIDENCE AND SEVERITY OF ARTICULAR CHANGES IN MICE OF VARIOUS STRAINS

Strain	Number of Animals	Articular Changes According to Grades, %				Age Distribution of Mice Examined, %			
		No Changes	Grade I	Grade II	Grade III	Group A 6–9 mo.	Group B 10–12 mo.	Group C 13–18 mo.	Group D 18+ mo.
Old Buffalo	15	52.7	26.7	7.3	13.3	00.0	13.4	60.0	26.6
C57	38	28.4	50.0	10.3	10.3	39.0	15.7	28.7	15.6
CBA	14	21.3	14.2	36.0	28.5	7.2	14.3	28.5	50.0
New Buffalo	24	33.4	46.0	12.4	8.2	00.0	33.3	41.7	25.0
A	32	37.4	34.0	15.8	12.8	21.8	18.7	40.5	19.0
D	23	26.4	22.8	26.4	26.4	39.0	13.0	22.0	26.0
C_3H	19	21.1	26.3	26.3	26.3	26.5	21.2	47.7	4.6

Total, 165 mice.

curve, which varies in different inbred strains and is genetically determined. He further concluded that inasmuch as in certain respects the genetic constitution of a closely inbred strain is comparable to that of an individual in a random population, the same conclusion applies to individuals.

Effects of hormones on the skeletal tissues. In growing mice, rats and guinea pigs, the time curve of the skeleton could be altered by administration of hormones and hormone-like substances, or by withdrawal of hormones normally produced in the body. Skeletal development and aging could thus be accelerated and intensified, or delayed and decreased (3).

During the first phase, the growth rate of the cartilage was intensified by the administration of bovine anterior hypophyseal extracts and of anterior pituitary growth preparation, of thyroid hormone, and of potassium iodide. However, this stimulation was followed by a premature cessation of the growth period. This may explain why gigantism did not occur in the animals treated with these substances. Moreover, the onset of the second phase, that of regression and ossification was accelerated. After administration of the anterior hypophyseal preparations, the intensification of hypertrophy and ossification (4–7) of the cartilage corresponded to the intensification of the proliferative processes in the cartilage. Potassium iodide had a similar, but milder, effect. Large doses of thyroxin and of parathyroid hormone, on the other hand, stimulated hypertrophy and regression of the epiphyseal cartilage more than its proliferation. Under these conditions, hypertrophy, regression and resorption proceeded so rapidly that proliferation was interfered with, and even stunting could result.

The duration of the second phase of skeletal development was shortened, and the onset of the third was accelerated by anterior hypophyseal preparations, thyroid hormone, parathyroid hormone and potassium iodide. Anterior hypophyseal preparations produced extensive perforations of the growth zones or even complete epiphyseo-diaphyseal union (fig. 10, 11). Still more effective in this respect was thyroxin that caused complete epiphyseo-diaphyseal union in all cases as early as in the second half of the first year of life. The stronger resorptive action of thyroxin may perhaps be due to the fact that it does not lose its effectiveness with prolonged administration, whereas the effect of anterior pituitary extract, of potassium iodide and of parathyroid hormone decreases with prolonged administration.

Syngenesio-transplants of anterior hypophyseal glands of mice to brother and sister animals increased the formation of bone (fig. 8) and diminished the growth rate of the cartilage, but they prolonged the growth period. The subsequent phase of regression of cartilage proceeded at an accelerated rate. Thus, in the end, development and aging of the skeleton were likewise accelerated.

The administration of estrogen inhibited both the growth of cartilage and the resorption of cartilage and bone (8, 9). However, in this case the growth period was not prolonged. The onset of regressive changes (fig. 7) in the epiphyseal cartilage was hastened and particularly the hyalinization and calcification of the cartilage were intensified. Thus, stunting of the animal may be the consequence of the administration of this hormone. In addition, the inhibition of resorption caused the persistence of the primary trabeculae and prevented their replacement by bone marrow. The excess of bone was increased by the additional deposition of new bone that encroached upon the marrow cavity and the inner layer of the shaft. The inhibition of resorption delayed the onset and progress of the third phase, but the regression of cartilage went on. As soon as the resorptive processes were resumed (8, 10), they proceeded rapidly; they led to perforations in the epiphyseal disk and to removal of the excess bone.

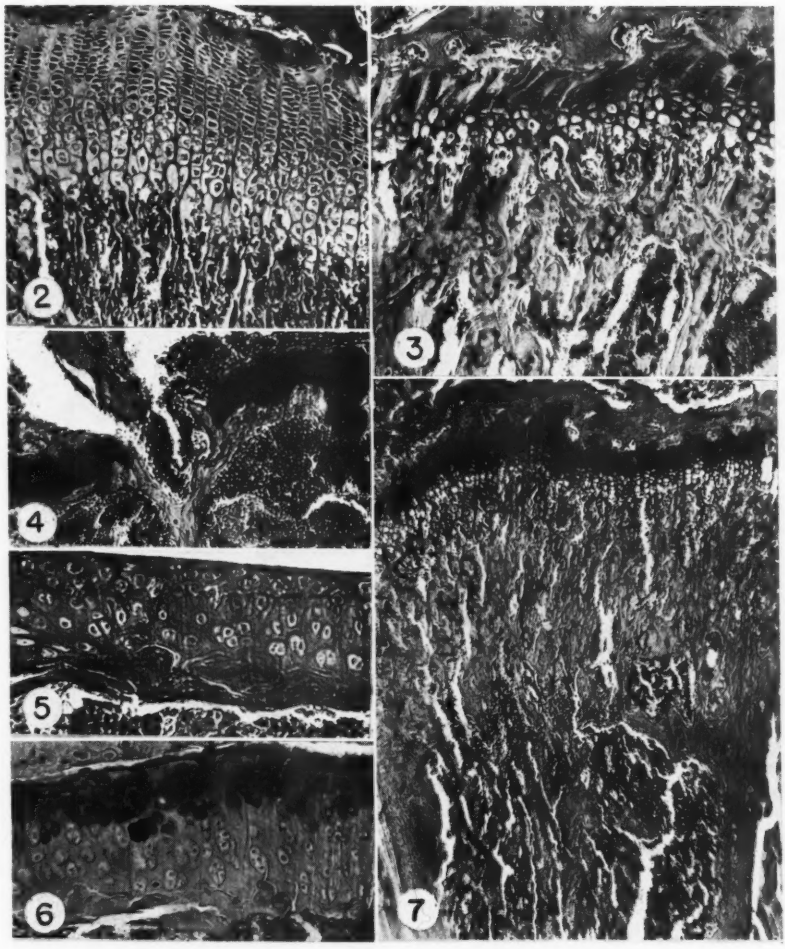

Fig. 2. Upper part of tibia of a C57 female mouse, 6 weeks old. Growth zone wide. Fig. 3. Upper part of tibia of a C_{3H} female mouse, 2 months old. Same magnification as in Figure 2. Growth zone distinctly narrowed and calcified, more and thicker bony spicules than in figure 2.

Fig. 4. Upper part of tibia of a C57 female mouse, 12 months old. Perforation of the densely calcified epiphyseal disk. Fig. 5. Articular surface of a joint of a C57 female mouse, 25.5 months old. Cartilaginous covering unchanged. Fig. 6. Articular surface of a joint of a CBA male mouse, 29 months old. Marked regressive changes in the articular cartilage.

Fig. 7. Upper tibia of a C57 male mouse, which starting at the age of 5 weeks had received 300 r.u. of estrogen-in-oil for 1 week. Growth zone heavily calcified and narrowed. Sub-epiphyseal trabeculae greatly thickened, here and there, interlaced with one another. Slight thickening of the shaft near the metaphysis. Shaft not thickened further distally.

Testosterone propionate acted similarly to estrogen, but its effects were milder. In particular, the inhibition of resorption was less accentuated (11, 12). Perforations in the epiphyseal disk occurred, therefore, much earlier and they were more extensive than after administration of estrogen (fig. 9).

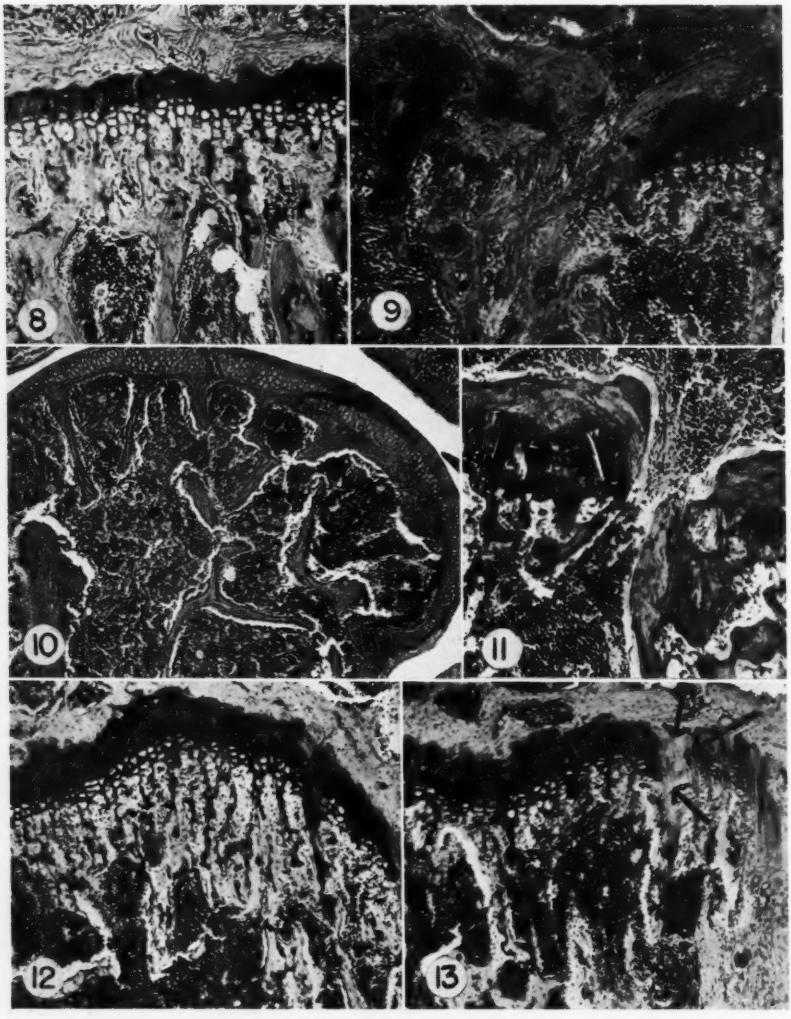

Fig. 8. UPPER TIBIA of a virgin A mouse, which at the age of 7.5 weeks had received 4 syngenesio-transplants of the anterior lobe of the hypophysis and which had been autopsied one week subsequent to transplantation. Growth zone narrowed and calcified. Cartilage cells decreased in size. Thick and dense trabeculae. Fig. 9. UPPER TIBIA of a female D mouse, 3 months old, which starting at the age of 4 weeks had received 0.25 mg. of testosterone propionate 3 times weekly for 2 months. Epiphyseal cartilage is being dissolved and replaced by bone and bone marrow. Beginning perforation of the epiphyseal disk.

Fig. 10. LOWER FEMUR of a male C57 mouse, which from the age of 2.5 months had been injected with 0.1 cc. of bovine anterior hypophyseal extract 5 times weekly. Epiphyseal disk resorbed. Complete epiphyseo-diaphyseal union has taken place. Fig. 11. LOWER FEMUR of a female D mouse, 3.5 months old, which had been injected with 0.1 mg. of thyroxin 3 times weekly for 2 months. Resorption has led to perforations of the calcified epiphyseal plate.

Fig. 12. UPPER TIBIA of a normal male C57 mouse, 16 weeks old. Calcified, inactive, narrow epiphyseal disk. Fig. 13. UPPER TIBIA of a male littermate of the animal shown in figure 12, which had been injected with 1.5 R.U. of growth hormone preparation 3 times weekly for 2 weeks. Same magnification as

Castration, as well as administration of progesterone, delayed the onset of the second phase and thus temporarily inhibited skeletal aging (3). Towards the end of the second and during the third phase, however, the resorptive processes proceeded more rapidly than usual and thus compensated for the delay in development and aging suffered during the preceding phase (fig. 14).

Effect of hormones on the aging processes in the joints. Prolonged administration of anterior pituitary hormone to growing animals accelerated the onset of the changes in the joints seen in untreated old mice. It also increased the severity and raised the incidence of these articular changes by about 40 to 50 per cent (5), as compared with the condition in untreated senescent mice. Hyperplasia, hypertrophy, fibrillation,

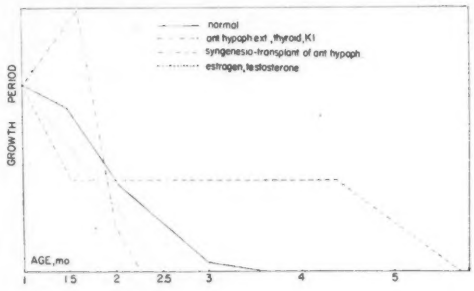

Fig. 14. DIAGRAM DEMONSTRATING the course of proliferation of cartilage (growth period) under normal conditions and under the influence of hormones in mice.

swelling and liquefaction of the articular cartilage cells and of the cartilaginous matrix took place (fig. 18). Resorption of cartilage and bone were intensified, and especially in guinea pigs, fibrosis and cyst formation were produced in the bone marrow. On the other hand, estrogen, testosterone, thyroid and parathyroid hormones increased the hyalinization of the cartilage, and, therefore, tended to decrease the incidence and severity of the spontaneous degenerative articular changes.

Combined action of hormones on the skeletal system. The age-promoting effect of estrogen on the skeleton could be modified by the simultaneous administration of other hormones. The results depended on the dose and on the duration of the experiment. In immature mice and guinea pigs, the simultaneous administration of progesterone decreased the aging effect exerted on the epiphyseal cartilage by estrogen alone, and the histological appearance of the growth zones resembled more closely that seen under normal conditions. Thus, progesterone apparently counteracted (3) the age-promoting effect of estrogen during the first periods of skeletal development. Under prolonged administration of estrogen and progesterone, no counteracting effect of the progesterone on the medullary bone formation was observed in mice (13). This finding might be correlated to the temporary nature of the effect of progesterone on the skeletal tissues. It seems as if the progesterone ceases to act, while the estrogen still continues to exert its effect on cartilage and bone.

The simultaneous administration of anterior hypophyseal extracts likewise modified the action of estrogen. In the early stages, the inhibition of growth of cartilage was less accentuated than after administration of estrogen alone. Likewise, the

in figure 12. Resorption of cartilage and bone further advanced than in figure 12. No stimulation of proliferation of the inactive calcified cartilage cells. Note the ossified plug of regressed cartilage, indicated by the arrows.

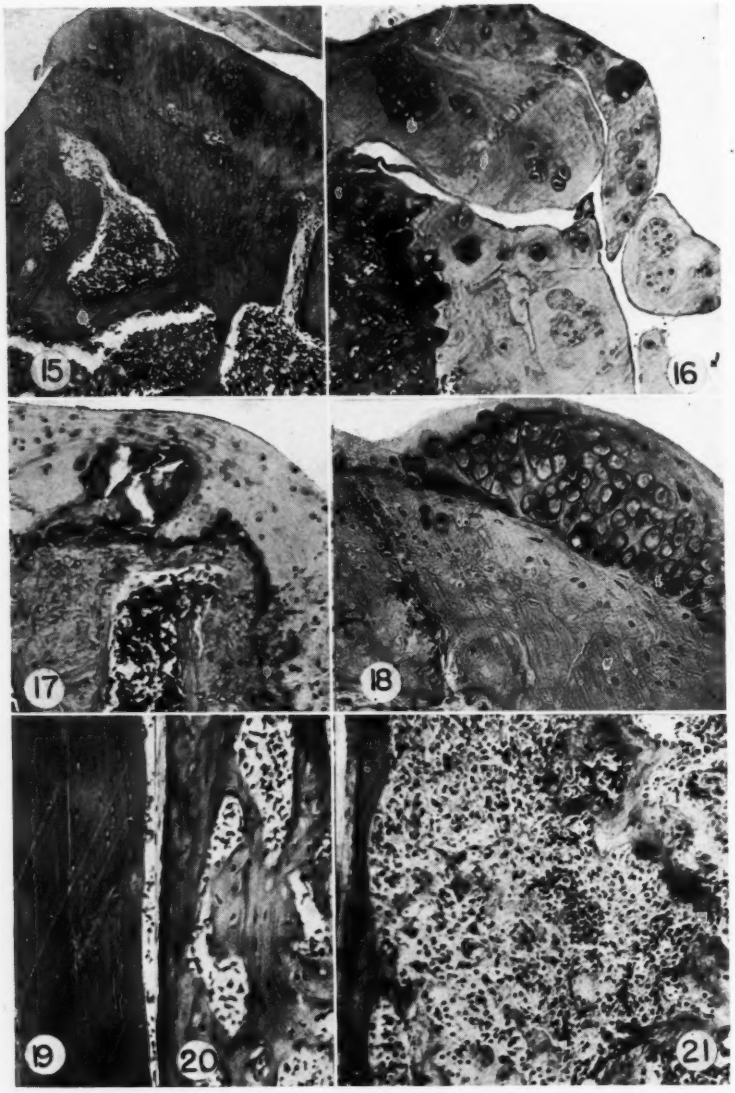

Fig. 15. KNEE JOINT of a female guinea pig weighing 750 gm. and about 1 year old at the beginning of the experiment. Injected with 4 cc. of bovine anterior hypophyseal extract for 60 days. Ulceration of the hyperplastic and hypertrophic articular cartilage with partial baring of subchondral bone. Capillaries of the bone marrow advance towards the surface of the joint. Fig. 16. EXPERIMENT SAME AS IN figure 15. Marginal cartilaginous outgrowth and formation of villi on the surface of the joint.

Fig. 17. EXPERIMENT SAME AS IN figure 15. Marked necrosis of the cartilage of the joint. Fig. 18. KNEE JOINT of a virgin A mouse, 14 months old, which had received 4 syngenesio-transplants of anterior pituitary glands at the age of 4 weeks. Hyperplasia, hypertrophy, some regression and resorption of the articular cartilage.

hyalinization and calcification of cartilage were decreased by this combination of hormones. Furthermore, increased softening and liquefaction of the cartilage indicated the influence of anterior pituitary hormone. Later, resorption of cartilage and bone was intensified and proceeded at a faster rate than after treatment with estrogen alone. Thus, anterior hypophyseal hormone diminished the effect of estrogen in mice, although not by a direct neutralization of the action of estrogen, but rather by its own peculiar action on the receptor tissues.

The combined administration of estrogen and thyroxin caused rapid resorption of cartilage and removed completely the excess bone produced by estrogen. Thus, thyroxin counteracted the inhibition of resorption and the new formation of bone called forth by the estrogen. Already towards the end of the first year of life, the histological picture resembled that seen after administration of thyroxin alone. Complete epiphyseo-diaphyseal union was established under these conditions. Thyroxin thus acted, at least partly, as an antagonist to estrogen, although both substances, if administered separately, accelerated skeletal aging.

The effect of estrogen could also be modified by the simultaneous administration of testosterone. In mice, the hormones interfered with each other at different times in a different way. At the early stage, each supported the other. The inhibition of both growth and resorption of cartilage was at first more accentuated than after treatment with either substance alone. At this stage, the testosterone may already promote resorptive processes, whereas the estrogen still continues to inhibit resorption. During this stage, therefore, the two hormones seem to counteract each other, as reported by Gardner and his collaborators (14). Finally, again the resorption did not make corresponding progress and an interlaced bony network could still be present after one year's treatment. In such instances, the effect of testosterone might have ceased altogether, whereas that of estrogen still continued.

In growing mice and guinea pigs, the aging effect of anterior hypophyseal preparations on the epiphyseal cartilage was intensified by simultaneous administration of thyroxin, and was temporarily increased by potassium iodide. On the other hand, the inhibiting effect which castration temporarily exerted on the process of aging of the epiphyseal cartilage, was counteracted by the administration of anterior pituitary extracts and transplants.

Significance of the age factor in the response of the skeletal tissues to hormones. The effects of hormones on the skeleton changed, if the substances were administered to adult and old animals. In most instances, adult and old animals were less responsive. Either they did not show any reaction at all, or at least larger amounts and longer administration of a hormone were required to elicit a reaction. This fact may be correlated with strain differences in the response to the administration of hormones. Generally, strain C57, exhibiting a slow rate of skeletal development and aging, reacted more readily to the administration of hormones than strain D showing a fast rate of skeletal development and aging. It is possible that the relatively advanced age of mice of strain D at the beginning of the experiment may partly account for the difference in the response to the various hormonal stimuli.

In mature adult animals, with intact hypophyses, that had ceased to grow before the treatment was begun, neither anterior hypophyseal preparations (15), anterior-

Fig. 19. SHAFT OF TIBIA of a normal virgin CBA mouse, 16 months old. Fig. 20. SHAFT OF TIBIA of a virgin CBA mouse, which starting at the age of 15 months had received 200 R.U. of estrogen-in-oil weekly for 6 weeks. Bony compacta soft and lamellated containing enlarged spaces filled with capillaries and bone marrow. Fig. 21. SHAFT OF TIBIA of a virgin C57 mouse which starting at the age of 12 months had received 200 R.U. of estrogen-in-oil weekly for 4 months. Compacta greatly thinned and replaced by osteoid connective tissue.

pituitary growth preparations, thyroxin, nor potassium iodide elicited proliferative processes in the epiphyseal cartilage. However, the course of such aging processes and developmental processes as were in progress at the beginning of the treatment, namely regression, ossification and resorption of the epiphyseal cartilage, could, to some extent, be accelerated by the administration of these substances (fig. 12, 13). In the cartilage of the joints, however, which normally does not calcify and which retains a certain degree of growth capacity, hypertrophy and particularly regressive changes could be intensified by the administration of anterior hypophyseal preparations. On the other hand, thyroxin and sex hormones injected into adult animals tended to increase the hyalinization of the cartilage as they did in growing animals.

In old animals, likewise, no proliferation of the remnants of the calcified inactive epiphyseal cartilage could be called forth by the administration of the various hormones and hormone-like substances. Even thyroxin had to be injected for a considerable length of time in order to produce increased resorption of the calcified cartilage.

Not only in adult, but even in old animals, the articular cartilage does not become completely inactive, and the cartilage of the joints retains some growth capacity even late in life. This may explain why administration of anterior hypophyseal preparations to old mice, and particularly to old guinea pigs, induced severe lesions in the senescent cartilage of the joints. These changes were comparable to those (16, 17, 18) seen in degenerative joint disease or osteoarthritis deformans in man. The same lesions could also be reproduced in ovariectomized animals treated with anterior hypophyseal preparations (fig. 15, 16, 17).

In contrast to conditions in growing mice, sex hormones and particularly estrogen, produced increased resorption of the shaft of the long bones and could lead to changes comparable to those seen in the course of osteoporosis. However, under these circumstances, the increased resorption was followed by new formation of bone. This latter change might be due to local factors in the tissues rather than to hormonal influences (fig. 19, 20, 21).

In conclusion it may be stated that, while the data which have been established so far give some insight into the influence of endocrines on the processes of growth development and regression and on aging in general, in the skeletal tissues, there are in all probability, in addition, genetic, nutritional and seasonal factors involved. These problems will have to be approached in future investigations.

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PROBLEMS RELATING TO THE ADRENAL CORTEX

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THE OBJECT of this paper is to review the anatomy, chemistry and physiology of the adrenal cortex with emphasis on the unsettled problems. Clinical observations and problems which concern therapy in patients are omitted.

ANATOMY

Morphology. The three zones of the adult cortex, glomerulosa, fasciculata and reticularis represent different phases in the life history of cortical cells. The cells proliferate from the capsule of the cortex, undergo further division in the zona glomerulosa and migrate through the zona fasciculata to the zona reticularis where the cell eventually degenerates (1, 2). It is not known whether the zones of the cortex are of more than morphologic significance. It has been claimed (3), on the basis of stain reactions, that the hormones of the cortex are secreted in the zone of the spongiocytes in the outer fasciculata, but there is little satisfactory evidence on the relationship of morphology to function. The use of stains to identify the position of steroid compounds in the different zones of the cortex may prove to be a valuable approach, but without complete knowledge of the specificity of stain reactions, they cannot provide proof of the identity of these compounds in tissues.

Howard (4, 5) has described the presence of an X zone in the cortex of the young mouse and rat and in the fetal cortex of the human. It lies between the zona reticularis and the medulla of the adrenal. Whether the X zone is a discrete tissue having a capacity for independent growth and function is not clear. It would be of interest to determine whether it would reappear during regeneration of the cortex in the enucleated adrenal of the young mouse. The age and sex relationships of the X zone indicate that it may exert an androgenic function. In mice it disappears at puberty but persists following castration. The administration of testosterone prevents the hypertrophy of the X zone following the castration of male mice (6). Gersh and Grollman (7) have reported degeneration of the X zone following administration of small doses of cortical hormone to immature or castrate mice, and its hypertrophy in response to the administration of thyroid substance. They deny that it has an androgenic function. In the young rat the presence of the X zone is associated with a transitory andromimetic activity (8). In the mouse there is evidence of extra-gonadal androgen production at a time when the X zone is well differentiated but following adrenalectomy and during treatment with 11-desoxycorticosterone acetate vesicle stimulation is maintained (9). The physiologic importance of the X zone, either as a source of androgens or as a source of other compounds, remains in doubt.

In most, if not all, species of mammals the adrenal cortex of the female is larger than that of the male. In the rodent the sex difference in size is marked but is largely abolished by castration at birth. The administration of estrogens (10) is followed by

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cortical hypertrophy, and androgens may cause some regression in size (11). Ingle (12) found that, in order to produce adrenal cortical atrophy in female rats, much larger doses of cortical extract were required than for males. Although the sex difference in size and resistance may possibly be explained in relation to the level of estrogens and androgens in the body, it is not known whether the functional activity of the cortex of the female is normally greater than that of the male. No evidence that such is the case has been obtained by comparing the life-maintenance requirements for cortical hormones by adrenalectomized male and female animals.

The significance of the lipoid content of the cortex in relationship to functional activity is not definitely established. The lipoid content is low when the functional activity of the cortex is thought to be low, is increased when the secretory activity is thought to be increased and depletion of lipoid is thought to be indicative of functional exhaustion (13) but proof is lacking. In animals under different types of stress, each of which is thought to stimulate the cortex to greater secretory activity, the amount of stainable lipoid in the cortex may vary widely. The problem is confused by the discrepancy in the reports of lipoid content as estimated by stains and by chemical analyses (14).

There is no known nervous control of the functional activity of the cortex. Al though innervation of the adrenal cortex has been frequently described, and generally accepted without question, Swinyard (15) has reported that the cortex of the cat and man is without neural connections with its cells. This finding has not yet been independently confirmed.

Pathologic changes occur in the cortex during certain vitamin deficiencies and in response to certain organic and inorganic toxic agents. In the case of pantothenic acid deficiency in rats, the hemorrhage and necrosis of the adrenal glands is very marked (16). The mechanism of degenerative processes in the adrenal is unknown. When an excess of cortical hormones is administered to normal rats the adrenal cortices undergo atrophy (17) but recover when the administration of the hormone is stopped. It is not known whether the prolonged administration of an excess of cortical hormones would cause permanent damage to the cortex.

Adrenal cortex, anterior pituitary relationships. Following removal of the anterior lobe of the pituitary the adrenal cortex undergoes extensive atrophy but can be restored to normal size by treatment with the adrenocorticotropic principle (18). When the normal animal is subjected to stress the adrenal cortices show hypertrophy (18). Cortical hypertrophy does not occur in the hypophysectomized animal (18). Cortical hypertrophy can be produced by the administration of adrenocorticotropic extracts, in either normal or hypophysectomized animals (18). When excessive amounts of cortical extracts, or certain steroids, are administered to normal animals the cortex undergoes atrophy (17). In the hypophysectomized animal in which the cortices are maintained at a normal size by treatment with the adrenocorticotropic principle, the administration of an excessive amount of cortical extract fails to induce cortical atrophy. These are the essential facts which indicate that the size of the adrenal cortex is regulated by the anterior pituitary and that changes in the size of the cortex, either regression or hypertrophy, is mediated by the adrenocorticotropic activity of the anterior pituitary. There are other experimental observations which may require a more complex explanation. Miller and Riddle (19) observed hypertrophy of the cortex of hypophysectomized pigeons following the administration of insulin. This observation that the cortex can adapt in the absence of the anterior pituitary requires confirmation and testing in other species. The cortex of the adult dog does not always atrophy following hypophysectomy (18). Ingle (20) administered large amounts of a

potent adrenocorticotropic principle to hypophysectomized rats in an attempt to stimulate regeneration of the enucleated adrenal. The results were negative and if confirmed will require explanation since regeneration of the cortex is thought to be controlled by the adrenocorticotropic hormone. Similarly, it would be of interest to determine whether autogenous transplants of the cortex could be stimulated to regenerate in the hypophysectomized rat by administration of the adrenocorticotropic hormone. It is possible that the amounts of adrenocorticotropic hormone required to stimulate regeneration are very much greater than are required to stimulate hypertrophy of the intact gland. Other activators of regeneration processes may be required. Golla and Reiss (21) extracted an adrenocorticotropic substance from pregnant mare's serum which causes hypertrophy of the cortex without affecting lipoid content thus suggesting the existence of two adrenocorticotropic principles. Astwood and Tyslowitz (22) have noted that the maximum size to which the adrenal cortex of the rat can be stimulated by means of adrenocorticotropic hormone differs greatly depending on the species from which the adrenocorticotropic hormone was derived.

The time and extent to which the adrenal cortex of the embryo is responsive to the anterior pituitary of the embryo and of the mother is unknown. Ingle and Fisher (23) reported that the adrenal glands of new-born rats from adrenalectomized mothers were heavier than those of new-born rats of normal mothers, thus indicating that the mechanisms responsible for compensating hypertrophy are active in the embryo; but negative results were obtained in a similar study by McKeown and Spurrell (24), thereby leaving this problem unsettled.

It is generally assumed that the atrophic cortex of the hypophysectomized animal has a functional activity lower than the normal but that the loss is not complete, since the hypophysectomized animal does not die of cortical insufficiency nor does it show the changes in electrolyte metabolism which characterize cortical insufficiency. But hypophysectomized animals have a low resistance to stress and show deficiencies in carbohydrate stores that can be attributed in part to a deficiency in the secretory activity of the cortex. Treatment of the hypophysectomized animal with active extracts of the adrenal cortex increases *a*), resistance, (25, 26, 27), *b*), the ability to perform muscular work (28) and c), raises the level of blood sugar and of liver glycogen (29). Those cortical steroids which are the most active in producing cortical atrophy (corticosterone, 11-dehydrocorticosterone, 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone) also have a marked effect on the metabolism of carbohydrate. The compound 11-desoxycorticosterone is relatively weak in its effect in producing cortical atrophy and in its effect on the metabolism of carbohydrate but is very active with respect to its effect on the metabolism of electrolytes and in maintaining the life of adrenalectomized animals. On the basis of these observations it has been suggested (18) that the regulatory function of the adrenocorticotropic principle is limited to the secretion of those compounds by the cortex which affect the metabolism of carbohydrate. The validity of this interesting interpretation has not been established.

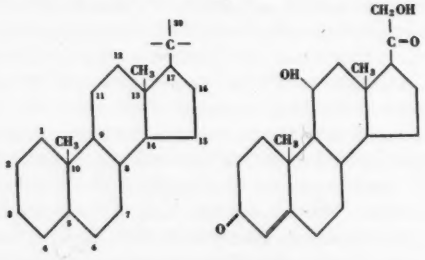
The specific change in the body tissues and fluids to which the adrenocorticotropic activity of the pituitary is responsive is unknown. Ingle and Kendall (30) maintained normal rats on diets which differed widely in the sodium and potassium content without inducing significant changes in cortex size. These experiments were carried out on the assumption that the adrenocorticotropic activity of the pituitary might be responsive to differences in the level of these electrolytes in the body. The negative results obtained may be regarded as having some weight against the hypothesis but do not constitute disproof. It would be desirable to determine whether or not wide

differences in the dietary intake of carbohydrate and protein would cause changes in cortex size on the assumption that the adrenocorticotropic activity of the pituitary may be responsive to the level of carbohydrate in the blood and tissues and to the physiologic need for gluconeogenesis from protein. The mechanism whereby the adrenocorticotropic principle affects the size and functional activity of the cortex is unknown and probably cannot be elucidated until the fundamental processes of growth are understood. In the rat, regression of the cortex can be induced by the administration of large doses of 11-desoxycorticosterone and to a lesser extent by progesterone (31) and testosterone (11). Each of these steroids is incapable of replacing the whole functional activity of the cortex. This raises a question as to whether a state of cortical insufficiency accompanies the atrophy caused by compounds which do not replace all cortical activity. Selye and Dosne (32) have shown that the administration of large amounts of desoxycorticosterone acetate inhibits but does not completely prevent cortical hypertrophy in the rat during the alarm reaction. These animals showed a resistance lower than the normal to non-specific damaging agents, as cold, trauma, exercise, formalin and atropine. The hypothesis that the subnormal resistance of these animals was due to an inhibitory effect of 11-desoxycorticosterone on the secretory activity of the cortex should be tested further by studying the resistance of animals having cortical atrophy produced by whole cortical extract or other adrenal steroids and by determining whether the subnormal resistance of rats treated with desoxycorticosterone can be raised to normal by treatment with the other cortical principles.

Most studies on cortical atrophy and hypertrophy have been carried out on the rat. It would be desirable to extend these studies to other species.

CHEMISTRY

Isolation and synthesis. Most of the information on the chemistry of the adrenal cortical steroids has been obtained by Reichstein (33) and his associates, Mason (34), Kendall and their associates and Wintersteiner and Pfiffner (35). More than 25 deriva-



CYCLOPENTANO-PHENANTHRENE SYSTEM

CORTICOSTERONE

tives of the cyclopentano-phenanthrene system have been isolated from the cortex of beef adrenals. This nucleus and the numbering of its carbon atoms is given in figure 1. Seven of these compounds have proven capable of replacing the functional activity of the cortex in one respect or another. They are corticosterone (fig. 1), 11-dehydrocorticosterone, 17-hydroxycorticosterone, 17-hydroxy-11-dehydrocorticosterone, 11-desoxycorticosterone, 11-desoxy-17-hydroxycorticosterone, and progesterone. The latter three substances of this group have been prepared by partial synthesis (36, 37, 38). Two active compounds have been prepared in the laboratory

which have not been isolated from the cortex. They are 11-desoxy-6-hydroxycorticosterone (39) and pregnene (5:6)-diol (3,21)-one (20), (36). The compounds 17-hydroxyprogesterone (40), estrone (41) and a weakly androgenic substance called adrenosterone (33) have been isolated from the cortex. The remaining compounds represent various combinations of 3, 4 and 5 oxygen atoms limited to substitutions at C- 3, 11, 17, 20, and 21. They have not been proven to possess biologic activity. Species differences in the nature and proportion of steroids have not been studied.

The compound 11-desoxycorticosterone is the most active compound isolated from the cortex in respect to life maintenance activity but it has not been shown to occur in sufficiently large amounts to account for a significant amount of the total life maintenance activity shown by cortical extracts. Kendall (42) has described the preparation of an amorphous fraction having life maintenance activity several times greater than 11-desoxycorticosterone. This claim has not yet been accepted by all of the workers in the field since the details of the biologic assay data have not been published and no other laboratory has succeeded in preparing an amorphous fraction which is significantly more active by weight than 11-desoxycorticosterone acetate. Ingle and Kendall (43) reported that the amorphous fraction was highly active in maintaining the ability of the rat to gain weight following adrenalectomy, but the periods of observation were short and there was no simultaneous comparison of the action of the amorphous fraction with that of the 11-desoxycorticosterone. Studies (44, 45) from Hartman's laboratories indicate the presence in the cortex of a principle which affects lactation in the rat, stimulates growth of the crop-gland of the pigeon, and an additional principle which causes the retention of sodium. The chemical isolation, identification and synthesis of substances occurring in the adrenal cortex which have thus far resisted attempts at crystallization remains the most important problem facing the organic chemist in the adrenal field.

The synthesis of corticosterone is a problem of practical importance since it has desirable biologic properties not possessed to any significant extent by 11-desoxycorticosterone. It is possible that a compound having an oxygen atom at C_{12} would manifest the biologic effects of corticosterone. The attachment of oxygen atoms and of side chains at various positions on the sterol nucleus, the preparation of higher homologs of 11-desoxycorticosterone by lengthening the side chain distal to C_{21} and the preparation of stereoisomers of these compounds are among the possibilities of obtaining new biologically active compounds.

In addition to steroid compounds, the cortex contains cholesterol, cholesterol esters, lecithin, lipids, ascorbic acid, glutathione, and other sulfur-containing compounds, carotene and a number of chemically unidentified nitrogenous compounds. It remains to be determined whether the cortex is a site for the storage or the elaboration of these substances, whether they are concerned with the production and transportation of the cortical steroids or whether they have any other functional significance in the gland.

Metabolism of the cortical steroids. The stages of the bio-synthesis of cortical steroids and their relationship to the other steroids of the body are not known. The possibility still must be considered that each steroid thus far isolated from the cortex may be either a precursor or a degradation product of some more active form of the hormone. The enzyme systems of the cortex are not known. Possibly the isolation of cortical enzymes and an elucidation of their rôle in the bio-synthesis of hormones would lead to useful methods for the laboratory synthesis of these compounds.

Raab (46) has reported evidence that the cortical steroids are chemically bound with epinephrine in the blood and tissues. The evidence is based upon a colorimetric

reaction which has not been proven specific for either epinephrine, the cortical steroids or for their combination. Neither the methods used nor the deductions made has been accepted as yet by other workers, therefore the question as to whether the cortical steroids are present in the blood in free or combined form remains open.

The manner in which the cortical hormones exert their biologic effects is a complete mystery. Do these compounds act as catalysts? Are they intimately involved in enzyme systems? Clowes, Davis, and Krahl (47) have suggested that the physical forces acting between certain polycyclic hydrocarbons and cholesterol in surface films may be sufficient to influence the physiologic function of cholesterol. An influence of double bonds and of the hydrophilic alcoholic hydroxyl groups on the physical orientation of these molecules is indicated. This work gives grounds for speculating about the possible importance of the physical effects of the cortical steroids in either causing or modifying their biologic effects.

Does intra-conversion of cortical steroids from one active form to another occur in the body in the absence of the adrenal glands? The whole problem of the intermediary metabolism of these compounds and the identification of their excretion products remains almost untouched. Weil and Browne (48) have reported the excretion of a biologically active cortin-like substance in human urine. Its chemical structure has not been established and little is known of its physiologic properties.

Relationship of structure to activity. The α:β-unsaturated ketone group and the α-ketol side chain are essential for optimal biologic activity of the cortical steroids. The conversion of 11-desoxycorticosterone to progesterone by removal of the oxygen atom at C_{21} greatly lowers the life maintenance activity. The reduction of the ketone group at C_{20} to a hydroxyl group causes a complete loss of activity. The reduction of the ketone group at C_3 to a hydroxyl group, with the consequent shifting of the ethylenic bond from the 4:5 to the 5:6 position, lowers but does not destroy the life-maintenance activity of 11-desoxycorticosterone. The reduction of the ethylenic bond lowers activity, but it cannot be stated with certainty that the loss of activity is complete. Numerous workers have found such compounds inactive at the dosage levels tested but Wells and Kendall (49) report that a C_{11} oxygenated cortical steroid, having a hydroxyl group in the C_3 position and completely saturated in the nucleus, had a definite effect on the carbohydrate metabolism of the rat; Kuizenga and Cartland (50) reported that a similar compound was effective in maintaining growth of adrenalectomized rats. The data indicating activity were not convincing in either case and further study is required.

The addition of a hydroxyl group to either position C_6 or C_{17} of the 11-desoxycorticosterone molecule lowers the life maintenance activity of the compound (51, 52). Similarly, the presence of oxygen, either as a ketone or a hydroxyl group, at C_{11} lowers the life-maintenance activity and the effect on electrolyte metabolism below that of 11-desoxycorticosterone. But these C_{11} oxygenated steroids (corticosterone, 11-dehydrocorticosterone, 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone) have a greatly increased effect on carbohydrate metabolism and important effects on resistance to stress which cannot be clearly defined (42). It has not been established with certainty that the quantitative activity of corticosterone differs from that of 11-dehydrocorticosterone. It is probable but not certain that the effect of these two compounds on carbohydrate metabolism is enhanced by the addition of a hydroxyl group at C_{17}. The compound 11-desoxy-17-hydrocorticosterone (52) does not affect carbohydrate metabolism. The life-maintenance activity of corticosterone and of 11-dehydrocorticosterone is definitely greater than that of 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone. In respect to the effect on electro-

lyte metabolism, corticosterone and 11-dehydrocorticosterone cause sodium retention and, according to a preliminary study by Thorn, Engel, and Lewis (53), 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone cause increased excretion of sodium.

The relative quantitative effects of the cortical steroids in maintaining the ability of the adrenalectomized rat to work (54) and in maintaining resistance to water intoxication (55) are found to parallel very closely the relative effects on carbohydrate metabolism.

The esterification of 11-desoxycorticosterone and of corticosterone is associated with an increase in biologic activity. It is not known whether this enhancement is caused by the change in the molecule or is the result of a prolonged period of utiliza-

TABLE 1. RELATIVE QUANTITATIVE EFFECTS OF COMPOUNDS WHICH HAVE BEEN PROVEN TO REPLACE T..E FUNCTIONAL ACTIVITY OF THE ADRENAL CORTEX IN ONE RESPECT OR ANOTHER

Compound[1]	Life Maintenance	Electrolyte Metabolism	Work	Carbohydrate Metabolism
Corticosterone	2	2	3–4	3–4
11-Dehydrocorticosterone	2	2	3–4	3–4
17-Hydroxycorticosterone	1	Na excretion ?	4	4
17-Hydroxy-11-dehydro-corticosterone	1	Na excretion ?	4	4
11-Desoxy-17-hydroxy-corticosterone	1–2	?	0	0
11-Desoxy-6-hydroxy-corticosterone	1–2	?	0	0
11-Desoxy-corticosterone	4	4	1	1
Pregnene (5:6)-diol (3, 21)-one (20)	2	?	0	?
Progesterone	1	1?	0	0
Amorphous fraction	+4?	?	2	?

[1] Efficacy gauged as 1, slight activity, to 4, marked activity.

tion either due to slower absorption or to a slower release because of the time required for hydrolysis.

Kendall (42) has claimed that the life maintenance activity of the amorphous fraction is greater than that of any known crystalline compound. In the absence of a presentation of the complete data upon which this conclusion is based, and without independent confirmation, the comparative activity of the amorphous fraction and the known crystalline compounds should not be regarded as settled. The other physiologic properties of the amorphous fraction are not clearly worked out. Wells and Kendall (56) have stated that its effect on electrolyte metabolism is less than that of 11-desoxycorticosterone. They studied its effect on the level of serum sodium and potassium in the blood of normal rats. Effects on electrolyte metabolism are not always reflected by changes in the serum as clearly as by changes in the renal excretion of electrolytes when dietary intake is known (57). Furthermore, no data were given on the amount by weight of the amorphous fraction administered. A comparison of the dosage of the amorphous fraction to the dosage of the crystalline compounds was made on the basis of dog units of activity. In other studies by Wells and Kendall (49) on the physiologic properties of the amorphous fraction, data on dosage by weight was also lacking, although it would seem to be essential for quantitative comparisons. Ingle and Kendall (43) studied the effect of the amorphous fraction on the ability of the adrenalectomized rat to work. Larger amounts were required than of 17-

hydroxy-11-dehydrocorticosterone. At present there is no proof that the most active life-maintenance principle of the cortex differs from 11-desoxycorticosterone in its physiologic effects. A satisfactory characterization of the biologic properties of the principles contained in the amorphous fraction must await the isolation of its compounds.

A summary of the biologically active cortical steroids and of their relative quantitative effects is given in table 1. This classification of qualitative and quantitative differences has been over-simplified.

The recent report by Linnell and Roushdi (58), that benzoyl carbinol and 3-hydroxyaceto-4-hydroxy-α:β-diethystilbene each possess cortical hormone activity, was not supported by convincing data. If these compounds prove to possess cortical hormone activity, the search for simple compounds with these biologic properties will become very important and the problem of the relationship of molecular structure to biologic activity will be greatly enlarged. It has been demonstrated that estrogens (59) including diethylstilbestrol (60) have an effect on carbohydrate metabolism similar to that of the C_{11} oxygenated steroids, but it is not clear whether the effect is direct or is mediated by other endocrine organs as the adrenal cortex or the anterior pituitary.

PHYSIOLOGY

In adrenal cortex insufficiency many functions of the body are changed from the normal. Since the presence of the cortex is essential for life, most of the physiologic changes are very similar to those shown by animals dying from, or severely damaged by, other unrelated causes. The separation of the primary and secondary changes due to cortical insufficiency has been difficult. Evidence obtained by studying the overdosage effects of pure compounds in intact animals has played an important part in establishing several of the known facts of cortical function. But it is probable that many of the physiologic properties of the cortical steroids and the mechanism of their action remain to be elucidated.

Growth and weight. The failure of the adrenalectomized animal to grow and to maintain normal weight can probably be attributed to the reduction in intake of food and to general physiologic abnormalities rather than to any intimate connection of cortical function with growth processes. Adrenalectomized animals will gain weight and grow without treatment with the cortical hormones, when a proper balance of dietary electrolytes is maintained and when other conditions are optimal (61, 62). Whole adrenal cortex extract, the amorphous fraction, 11-desoxycorticosterone, corticosterone, 11-dehydrocorticosterone, 11-desoxy-6-hydroxycorticosterone and pregnene (5:6)-diol (3, 31)-one (20) are each capable of maintaining the ability of adrenalectomized rats to gain weight. Wells and Kendall (56) have reported that 11-desoxycorticosterone causes an increase in the rate of growth of normal rats. It was not determined whether this was a true acceleration in growth or was due to increased water retention.

Kuizenga and Cartland (50) reported growth of adrenalectomized rats treated with 17-hydroxy-11-dehydrocorticosterone whereas Wells and Kendall (63) and Ingle (64, 65) have observed either a suppression of growth or an actual loss of weight in rats treated with this substance. This discrepancy may be due to differences in experimental conditions and in dosage, but further study is indicated. When large amounts of 17-hydroxy-11-dehydrocorticosterone are administered to normal rats (65) on a constant intake of food, the weight loss may be very rapid and out of proportion to the negative nitrogen balance. Undetermined factors such as dehydration may be involved. Grollman and Firor (66) described the stunting of the growth of rats by

chronic cortical insufficiency which persisted when the intake of cortical extract was increased. Growth was then stimulated by the administration of anterior pituitary growth principle, thus indicating that during chronic cortical insufficiency the production of growth principle by the anterior pituitary is permanently impaired. This work has not been independently confirmed. Growth of the hair of adrenalectomized rats is increased above that of normal rats. The reason is unknown. The healing of wounds is retarded during cortical insufficiency. It is not known how the healing processes are related to primary and secondary changes of cortical insufficiency.

Salt and water metabolism and the circulation of blood. Animals dying from any deficiency disease show circulatory inadequacies for variable periods prior to death. In the case of adrenal cortex insufficiency it has been established that some circulatory changes are secondary to changes in electrolyte metabolism. In uncomplicated adrenal cortex insufficiency the excessive loss of sodium and chloride ions and retention of potassium ions by the kidney leads to an accompanying increase in the loss of water from the blood and tissues so that dehydration and an eventual circulatory collapse occur (67, 68, 69). The primary importance of electrolytes is indicated further by the beneficial effect of a high sodium-low potassium intake in adrenalectomized animals (70) and by the powerful effect of certain adrenal cortex extracts and steroids on sodium retention and potassium excretion (71, 72). But the conditions of adrenal insufficiency can be modified so that no more than a part of adrenal cortex physiology can be explained in terms of altered kidney threshold to electrolyte excretion.

For the purpose of discussion, the principle factors involved in the distribution of salt and water are summarized as follows.

1. Intake
2. Rate and completeness of absorption from the gut
   - A. Permeability
     - a. selective activity of membranes
     - b. vital activity of membranes
   - B. Physical Forces
     - a. osmotic pressure
     - b. intra-intestinal pressure
     - c. surface tension
   - C. Peristalsis
   - D. Pumping action of villi
   - E. Blood-flow
3. Kidney
   - A. Blood-flow and pressure
   - B. Tubular absorption
   - C. Anti-diuretic principle of the pituitary
4. Transudation between blood and tissues
   - A. Capillary permeability
     - a. passive
     - b. vital?
     - c. selective
   - B. Pressures
     - a. osmotic
       - (1) crystalloids
       - (2) colloids
     - b. hydrostatic
       - (1) within capillary
         - (a) capillary blood-flow
         - (b) capillary tone
       - (2) outside capillary
         - (a) turgor pressure
         - (b) deformational pressure
   - C. Blood-flow
     - a. rate
     - b. volume
     - c. distribution
   - D. Muscular activity
5. Bound salt and water?
6. Vomiting
7. Sweat glands
8. Insensible loss of water

During cortical insufficiency the intake of food and water is reduced, consequently lowering the intake of electrolytes. On the other hand, the appetite for sodium chloride is increased in adrenalectomized rats (73) and the taste threshold for detection of differences in sodium chloride concentrations is significantly decreased (74). Quantitative studies on other laboratory animals are desirable. Adrenalectomized rats also make a small increase in their intake of potassium salts during voluntary selective feeding (73). Since an intake of potassium lower than normal is beneficial, this observation on a voluntary increase in potassium intake should be studied further.

There is little information on the rate of absorption of electrolytes and water from the gut of adrenalectomized animals. Denis and Wood (75) demonstrated a decrease in the rate of absorption of sodium, potassium and chloride from loops of ileum in adrenalectomized dogs maintained on high sodium, high bicarbonate, low potassium, diets. Clark (76) observed that the rate of intestinal absorption of sodium chloride was slower than normal in adrenalectomized rats which were maintained in a state of apparent good health on Rubin-Krick-salt drinking fluid. Stein and Weytheimer (77) have also shown that the absorption of inorganic ions by the intestinal tract is not normal. It cannot be deduced with certainty that these observed changes in intestinal absorption were due to primary changes of cortical insufficiency. There is little data in the literature on the completeness of absorption of electrolytes from the gut of adrenalectomized animals. During the diarrhea of severe cortical insufficiency significant amounts of water and electrolytes may be lost in the stools but this is a terminal condition.

The site of action of cortical steroids on kidney function is believed to be confined to the tubules. This is not known with certainty but there is no contrary evidence. The mechanism of the action is unknown. Is it an effect that is specific for kidney tissue or is the effect more general? If the effect is more general it at least may be said that the kidney is the most important site of action, for the changes in kidney threshold to sodium, chloride and potassium may determine whether the animal will live or die. The relationship of adrenal cortex function to the kidney control of electrolyte and water balance is more complex than is usually described. Anderson, Joseph, and Herring (78) have shown that the clinical condition of the adrenalectomized rat determines to some extent the functional capacity of the kidney. Untreated adrenalectomized rats which were maintained in good clinical condition by a high sodium chloride intake were better able to retain sodium and excrete potassium than were animals on a lower sodium chloride intake. These workers (79) have shown further that the defect in the functional capacity of the kidney is not limited to its failure to retain sodium when retention is needed. Adrenalectomized rats were incapable of excreting as much sodium as normal rats, when excessive amounts of sodium chloride were administered, and they were found to succumb at high levels of intake of sodium chloride which were tolerated by normal animals. Similarly, Kottke, Code and Wood (80) found that the maximum ability of the kidney in adrenalectomized dogs for concentrating chloride was less than that of intact animals. Thorn, Engel, and Lewis (53) reported that the cortical steroids with a hydroxyl group at C_{17} cause an increased excretion of sodium and chloride in either normal or adrenalectomized animals. Certain estrogens and androgens have the property of causing retention of sodium by the kidney but are without benefit to adrenalectomized animals (81). The adrenalectomized opossum fails to show an increased excretion of sodium and chloride and may succumb with values of serum sodium and chloride which are higher than the normal (82). There is no concept of cortical control of kidney thresholds for electrolyte excretion which satisfactorily explains these and other experimental observations. There are insufficient data on the blood-flow of the kidney during cortical insufficiency. It seems probable that secondary circulatory changes in the kidney may modify its function during cortical insufficiency.

Ragan et al. (83) found that diabetes insipidus can be induced in normal animals by the prolonged injection of 11-desoxycorticosterone. This is thought to be a compensatory response on the part of the kidney to prevent excessive retention of sodium, but the mechanism is not understood. Corey and Britton (84) have evidence for an antagonism between the anti-diuretic principle of the posterior pituitary and the

cortical hormone. The diabetes insipidus which follows removal of the posterior lobe is abolished when the anterior lobe is also removed but it can be re-induced by the administration of large amounts of 11-desoxycorticosterone. It is suggested that the effect of the anterior lobe on the renal excretion of water is by the adrenocorticotropic principle and is mediated by the adrenal cortex. Schweizer et al. (85) disagree with this deduction since the adrenocorticotropic principle and cortical extracts were found by them to be without effect on the excretion of water by hypophysectomized rats. Although 11-desoxycorticosterone was effective, larger amounts were required than are likely to be produced by the cortex. It is not yet established that the normal physiology of the adrenal cortex includes an effect on the renal excretion of water other than that mediated by its effect on electrolytes.

During the usual course of adrenal cortex insufficiency the excessive loss of sodium and the accompanying diuresis may be regarded as a primary break-down leading to hemoconcentration and the eventual development of shock, but it is possible to set up experiments in which the circulatory collapse can be dissociated from kidney dysfunction. Not only can the animal maintain circulatory adequacy in the presence of very low values for serum sodium and chloride (86), but a circulatory collapse can co-exist with normal levels of electrolytes in the blood serum (87). Internal shifts in fluid and electrolytes past capillary walls and cell membranes of tissues may occur without being dependent on kidney function, and cortical extracts and certain steroids are capable of reversing these changes without other therapy. What are the specific mechanisms of hemoconcentration during states of cortical insufficiency shock? Are changes in capillary permeability primary, and if so, are they due to pathologic changes or can the change be characterized as functional? What is the relative importance of the rôle played by changes in osmotic and hydrostatic pressures in causing the fluid loss? Is capillary atony a factor as indicated by Swingle et al. (88)? Can the presence or absence of cortical principles affect the movement of intra-cellular and intercellular electrolytes and water independently of an effect on the blood and vessel walls? The observation (89) that the cortin-treated adrenalectomized animal is capable of recovery without the administration of either electrolytes or fluids is important. The mechanisms by which changes resulting in shock are reversed seem difficult to explain on a basis of the known osmotic and hydrostatic pressures. Can capillary membranes secrete water and electrolytes into the blood stream by performing work and have the cortical principles an effect on this mechanism if it exists?

Is the increase in blood serum potassium which occurs during cortical insufficiency due entirely to the raised kidney threshold and the consequent retention of potassium, or is there an additional loss of potassium from cells? Ingle, Nilson and Kendall (90) reported that the administration of cortical extracts to adrenalectomized nephrectomized rats suppressed the rise in serum potassium but in these experiments there were several possible sources of experimental error which were not recognized at the time their results were reported. Negative findings were reported by MacKay, Bergman and MacKay (91) and by Hartman and Dubach (92) in similar experiments. It is claimed by Cantarow and Rakoff (93) that the rate at which sodium and chloride enter the peritoneal cavity of the dog following the intraperitoneal injection of glucose is greatly increased by 11-desoxycorticosterone, progesterone, and dietylstilbestrol. If these observations are confirmed the effects of other steroids should be studied.

It has been suggested that the effect of the cortical principles on both kidney function and on extra-renal shifts of water have in common a specific effect on membrane permeability, but the evidence has been indirect and unsatisfactory. It would

be desirable for men who are outstanding investigators in the field of membrane permeability to come to the assistance of adrenal physiologists in studying this problem. An interesting beginning has been made by Netsky and Jacobs (94), who demonstrated striking effects of certain steroids on the permeability of the mammalian red blood cell. The relationship of specificity of steroid structure to permeability effect has not yet been established.

Other avenues such as the sweat glands and lungs for the loss of electrolyte and water from the body during cortical insufficiency, are probably of minor importance. Loss of chloride by vomiting may become serious during the later stages of cortical insufficiency, but this is a secondary factor. It is claimed that sodium chloride is stored, especially in the skin. The amount stored or lost during cortical insufficiency has not been studied.

Carbohydrate and protein metabolism. Adrenal cortical insufficiency is characterized by hypoglycemia and depletion of the tissue carbohydrate stores, especially during fasting (95). These changes are striking in most laboratory animals but not in the dog in which hypoglycemia is usually seen only in the terminal stages of insufficiency. In animals made diabetic by the removal of the pancreas (96) or by the administration of phlorhizin (97) the glycosuria is decreased by removal of the adrenals. In adrenalectomized animals there is a decrease in the formation of carbohydrate from protein as evidenced by the accompanying reduction of urinary non-protein nitrogen. The adrenalectomized animal is abnormally sensitive to insulin. These changes can be reversed by treatment with cortical extracts, corticosterone, 11-dehydrocorticosterone, 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone. Not only can the deficiencies in carbohydrate metabolism be restored to normal levels, but the carbohydrate stores of the blood and tissues can be increased above the normal in either intact or adrenalectomized animals (98). The diabetogenic effect of cortical extracts and of certain steroids is so marked that partially depancreatized rats which do not show any spontaneous glycosuria can be made to excrete all the available carbohydrate of the diet during the administration of these cortical principles (99). The observation by Ingle (100) that normal rats can be made severely diabetic by the administration of large amounts of 17-hydroxy-11-dehydrocorticosterone has not yet been independently confirmed. Either normal or adrenalectomized animals become abnormally resistant to the convulsive effect of insulin during treatment with the C_{11} oxygenated cortical steroids (101, 102).

For the purpose of discussion, the principle factors involved in carbohydrate metabolism are summarized as follows:

1. Intake of food
2. Rate of absorption from the gut
   - A. Phosphorylation or other unidentified chemical changes
   - B. Permeability
     - a. selective activity of membranes
     - b. vital activity of membranes?
   - C. Physical forces
     - a. osmotic pressure
     - b. intra-intestinal pressure
     - c. surface tension
   - D. Pumping action of villi
   - E. Peristalsis
   - F. Blood flow
3. Rate of deposition in tissues
   - A. As glycogen
   - B. As fat
4. Rate of glycogenolysis
5. Gluconeogenesis from protein
   - A. Mobilization of endogenous protein
   - B. Deamination of amino acids
   - C. Conversion of deaminized acids to glucose
6. Rate of utilization
   - A. Rate of insulin secretion by pancreas
   - B. Direct inhibitory effect on insulin
   - C. Direct effect on carbohydrate utilization
7. Kidney threshold

One of the secondary changes of cortical insufficiency is loss of appetite so that

the amount of carbohydrate available from food is usually reduced. In adrenalectomized rats (103) and dogs (70) food intake can be restored to normal levels by an optimal intake of electrolytes. The survival of adrenalectomized animals in relationship to the carbohydrate and protein content of diet is not established. Richter (104) observed that the self-selected diet of adrenalectomized rats has a content of carbohydrate that is lower than the normal.

During cortical insufficiency the rate at which glucose is absorbed from the intestinal tract is slower than the normal rate. Verzar (105) postulated that the cortex is essential for phosphorylation processes and that inhibition of the phosphorylation of glucose during cortical insufficiency is responsible for the reduced absorption of glucose. The numerous failures to confirm this hypothesis would seem to make it untenable. Secondary physiologic changes during adrenal cortical insufficiency may be responsible for slow intestinal absorption. The absorption rate of glucose has been reported as normal in adrenalectomized rats maintained on a high intake of sodium chloride (106, 107). The problem has usually been investigated in rats and studies should be extended to other species.

It has been reported many times that the rate at which blood glucose is deposited as glycogen in the tissues is slowed during cortical insufficiency. Long, Katzin and Fry (98) and Anderson, Herring and Joseph (108) have shown that the adrenalectomized rat maintained on a high sodium chloride intake is capable of depositing liver glycogen at a normal rate. Corey and Britton (109) reported that the addition of cortical extract to a solution used to perfuse cat liver gave a marked acceleration in the rate of glycogen deposition. If this work is confirmed the cortical steroids should be examined for this property. Kendall (42) attributes the effect of the cortical principles on deposition of liver glycogen to the activation of the enzymes involved but there is little evidence on the nature of the relationship of the cortical principles to enzyme systems.

In adrenalectomized animals the rate of gluconeogenesis is reduced during fasting, but can be increased above the normal rate by the administration of large amounts of certain cortical principles. These changes are easily demonstrated when the animal is dependent on endogenous protein as a source of new carbohydrate, but it is less certain that there is a definite effect of the cortical principles on the conversion of fed protein. Long, Katzin and Fry (98) have suggested that the effect of cortical principles is on the mobilization of endogenous protein. Wells and Kendall (49) report that the phlorhizinized adrenalectomized rat forms glucose from fed protein at a normal rate but Lewis et al. (110) found that the formation of glucose from lactic and pyruvic acids and alanine in the adrenalectomized, phlorhizinized rat was subnormal. Kendall (42) suggested that secondary changes in the experimental animals may have been responsible for the observed deficiency. Koepf et al. (111) demonstrated that liver slices from rats treated with cortical extracts converted lactic and pyruvic acids to carbohydrate at a rate greater than the normal. Changes in isolated tissues provide evidence, but not proof, of the nature of physiologic processes in intact animals. Ingle and Thorn (99) observed that partially depancreatized rats on a constant diet failed to show a decrease in the excretion of non-protein nitrogen following adrenalectomy, although the excretion of glucose was abolished. Evans (112) reported that in adrenalectomized rats the deamination of dl-alanine proceeds at a normal rate. Russell and Wilhelmi (113) observed that kidney slices from adrenalectomized rats form less than normal amounts of carbohydrate when incubated with dl-alanine, l (+) glutamic acid, and α-ketoglutaric acid and that normal amounts were formed from succinic and pyruvic acid.

There is evidence that some of the cortical principles inhibit the utilization of carbohydrate. The R.Q. of the adrenalectomized animals and of patients having Addison's disease is high and it can be depressed by the administration of cortical extracts and certain steroids (98, 110, 114). There is no known primary defect in the ability of the adrenalectomized animal to oxidize carbohydrate. In force-fed, partially depancreatized (99) and in normal (100) rats made diabetic by the administration of 17-hydroxy-11-dehydrocorticosterone, the amount of glucose excreted was much greater than could be accounted for on the basis of the amount of non-protein nitrogen excreted. Under the influence of this steroid, partially depancreatized rats on a constant intake of food, which do not excrete glucose during the control period, can be made to excrete all of the available carbohydrate of the diet during the injection period. Similarly, the decrease in the glycosuria of the diabetic rat following adrenalectomy is greater than can be accounted for on the basis of decreased gluconeogenesis from protein (99), thus indicating that the animal has become able to utilize more carbohydrate. The manner in which the cortical principles inhibit the utilization of carbohydrate is not known. Is it a direct effect or is it mediated by an inhibition of insulin secretion or by a direct blocking of the action of insulin? Haist and Bell (115) reported that the insulin content of rat pancreas was not modified by the administration of cortical extract, but they did not test large doses and they did not examine the single compounds known to be glycotropic and diabetogenic. Grattan and Jensen (101) found that those cortical steroids which are oxygenated at C_{11} prevent insulin convulsions in mice and that the liver glycogen level that is higher than the normal in these treated mice may account for the 'anti-insulin' effect. The adrenalectomized animal is remarkably sensitive to insulin and it is not clear that this increased sensitivity is due solely to the depleted carbohydrate stores. Wells and Kendall (49) have suggested that the C_{11} cortical steroids block the action of insulin, but proof is lacking. Wells and Kendall (49) state that the cortical steroids have an inhibitory effect on hypoglycemic convulsions independent of their effect on carbohydrate metabolism, since treatment prevented the convulsions from occurring at a time when the blood sugar had not been raised above that of untreated animals. Such evidence is not convincing, for the level of blood glucose does not necessarily reflect all changes in the metabolism of carbohydrate. It is known that hypoglycemic convulsions are not determined by the level of blood glucose *per se* but there is good reason to believe that some phase of the distribution or utilization of carbohyrate is the dominant determining factor.

It is not yet known how the changes from normal in the absorption, deposition, formation and utilization of carbohydrate are related. In rats made severely diabetic by pancreatectomy or by treatment with estrogens (60) there is an increase in protein catabolism similar in extent to that observed in rats made diabetic by the administration of 17-hydroxy-11-dehydrocorticosterone (99). Is the inhibition of carbohydrate utilization primary to the increase in gluconeogenesis? Studies on the fasting animal and on the formation of new carbohydrate by liver slices would indicate that the liver is the site of a primary action of the hormone. But the possibility must be considered that the response of the liver is compensatory for the inhibitory effect on utilization of carbohydrate by the peripheral tissues. If the hormone has effects on tissues other than the liver it should be possible to demonstrate an effect on the utilization of glucose by the hepatectomized animal. Selye and Dosne (116) carried out such a study with negative results. They used relatively small amounts of cortical extract and did not test the more active diabetogenic principles. Studies on liver glycogen levels as affected by the cortical steroids have been confined to non-

diabetic animals. In animals with severe pancreatic diabetes the liver glycogen is usually very low. What is the liver glycogen level in animals made severely diabetic by means of the cortical steroids? Can a state of permanent diabetes be induced in intact animals by the prolonged treatment with cortical steroids? There are now a number of studies demonstrating an effect of estrogens on carbohydrate metabolism. Not only are they effective in raising the liver glycogen of normal rats (117) but they are diabetogenic (59, 60). Are the cortical steroids and estrogens similar in their mode of action on the metabolism of carbohydrates?

Kelsche and Kendall (118) reported that unilaterally and bilaterally adrenalectomized dogs were thrown into negative nitrogen balance by the administration of thyroxin and that the administration of cortical extracts restored a positive nitrogen balance. These findings seem inconsistent with the fact that the cortical principles themselves tend to increase protein catabolism and they require further explanation.

Metabolism of fat. Changes from normal in the intestinal absorption of fat by adrenalectomized animals has been both affirmed (119) and denied (120, 121). Adrenalectomy causes a decrease in the severity of experimental ketonuria but MacKay and Wick (122) explain this as due to a raised kidney threshold for the excretion of ketone bodies and indicate that there is no established effect of cortical insufficiency on the rate of ketosis. The production of fatty livers in rats by the injection of pituitary extracts is abolished by adrenalectomy (123, 124). This finding has not yet been explained. Ingle and Thorn (99) reported the production of severe ketonuria in partially depancreatized rats made severely diabetic by the administration of 17-hydroxy-11-dehydrocorticosterone. But the ketonuria occurred only in association with glycosuria of a severity which is accompanied by ketonuria in the rat regardless of the type of diabetogenic agent used. The cortical steroids do not cause ketonuria when tested in the fasting normal rat (125).

The level of cholesterol in the blood is usually found to be elevated during cortical insufficiency and Villela (126) found it to be depressed below normal in the blood of non-adrenalectomized guinea pigs treated with 11-desoxycorticosterone. The significance of these observations is not known.

Resistance to stress. The adrenalectomized animal is abnormally sensitive to every type of stress. The same is true for many other deficiency diseases. It is difficult to explain the lowered resistance of adrenalectomized animals in terms of the known effects of the cortical principles on salt and water metabolism and carbohydrate metabolism. Cortical extracts effect the survival time and work performance of the completely eviscerated rat in the presence of adequate glucose when the kidneys and the liver are absent (127). Partially depancreatized rats which excrete a large amount of glucose daily on a constant diet usually give the appearance of normal health and vigor when their adrenal glands are present. When such animals are adrenalectomized and maintained on 2 mg. daily of 11-desoxycorticosterone (a salt and water factor) the glycosuria disappears but can be re-induced by the administration of small amounts of diethylstilbestrol. With the reappearance of a mild glycosuria and elevated blood sugar these animals rapidly become ill and die. Under similar conditions, diabetic adrenalectomized rats maintained with cortical extract exhibit a much greater resistance. This has been observed repeatedly by Ingle (20) and is a phenomenon difficult to explain as due to failure of the sodium and carbohydrate factors. Adrenal shock may occur without sodium loss by the kidneys and without hypoglycemia (128). There are other experimental observations on adrenalectomized animals which are equally difficult to explain. Is the vascular mechanism the site of some additional primary deficiency, or is the increased sensitivity to shock only one manifestation of

a deficiency which is general for all tissues? Zwemer (129) has suggested that the release of potassium into the blood stream is a factor of major importance during stress. The abnormal sensitivity of the adrenalectomized animal to potassium is well established and the potassium level in the extra-cellular fluid may be one of the factors determining the rate of development of cortical insufficiency, but the mechanism of the increased sensitivity to potassium and all other toxic agents is unexplained. The abnormal retention of potassium by adrenalectomized animals usually does not exceed amounts which are easily tolerated by non-adrenalectomized animals. Kendall and Ingle (130) reported that the adrenalectomized dog maintained without hormone on a high sodium chloride diet is capable of acquiring a tolerance to a dietary intake of potassium that is higher than the normal when the increases in potassium intake are made very gradually. When the same extent of increase was made during a short period of time symptoms developed. If these observations can be confirmed, the adaptation to potassium levels by the adrenalectomized animal should be studied more extensively.

Selye (131) suggested that the similarity between the symptoms of cortical insufficiency and those exhibited by non-adrenalectomized animals during the stress, the alarm reaction, is due to a state of relative cortical insufficiency in the non-adrenalectomized animals. But cortical insufficiency can also be regarded as another means of causing non-specific damage to the organism. From this standpoint the secondary symptoms of cortical insufficiency may be regarded as an alarm reaction rather than to assume that any alarm reaction is a state of relative cortical insufficiency. Although it is very probable that under certain conditions of stress the cortex is incapable of meeting the functional requirements for its hormones, thus making symptoms of damage more likely to occur, it has not been proven that a state of functional cortical insufficiency is an essential condition for the manifestation of the alarm reaction. In either partially depancreatized (99) or normal rats (100), changes which are similar in some respects to those of the alarm reaction can be induced by the administration of large amounts of either cortical extract or of 17-hydroxy-11-dehydrocorticosterone. The pathologic changes occurring during the alarm reaction cannot be prevented by treatment with cortical principles, although in some experimental situations resistance can be increased. The extent to which the resistance of non-adrenalectomized animals can be increased by treatment with the cortical hormones is not clearly established and the elucidation of this problem is needed as a basis for the possible clinical use of these principles in treating traumatic, surgical and other types of wartime shock. Remington et al. (87) have observed qualitative differences in the effect of 11-desoxycorticosterone and corticosterone on resistance to different types of shock in adrenalectomized dogs. The relative efficacy of the different cortical steroids for resistance to different types of stress has not yet been studied in detail. One of the oldest theories of cortical function is that it is concerned with the catabolism of toxins. Knowledge of the relative rate of catabolism of toxins in adrenalectomized and normal animals and of the effect of the different cortical hormones on rate of detoxification is meager. Rose and Browne (132) and Wilson (133) have observed that the cortical principles restore to normal the deficient histaminase activity in adrenalectomized animals. Whether the adrenal cortex has a specific effect on the catabolism of histamine as suggested by Selye (131) is not known.

The adrenal cortex increases in size in response to stress. Can physiologic changes due to increased secretory activity of the cortex be demonstrated? Evans (97) found that exposure of rats to low atmospheric pressures caused an increase in liver glycogen. The response did not occur in the absence of the adrenal glands. Since the

administration of cortical principles causes an increase in liver glycogen and blood sugar of adrenalectomized animals at either normal or low atmospheric pressure (134), and since the cortex is increased in size (135) when the animal is subjected to low pressures, it is reasonable to suggest that the increased liver glycogen is caused by an increased production of cortical hormones. But the failure of a response to occur in the absence of an organ is not proof that the organ mediated the response. Langley (136) has shown that adrenalectomized rats treated with sub-glycotropic amounts of adrenal cortical extract also show an increase in liver glycogen during acute anoxia. A similar situation exists with respect to the diabetogenic effect of diethylstilbestrol which also causes cortical hypertrophy and an increase in liver glycogen (117). The diabetogenic response to diethylstilbestrol is abolished by adrenalectomy but when a sub-diabetogenic amount of cortical extract is substituted for the cortices, and its intake is kept constant throughout the control and experimental periods, glycosuria again occurs when diethylstilbestrol is administered (20). It is quite possible that the increase in the size and secretory activity of the cortex during stress is only sufficient for the maintenance of homeostasis and that abnormal changes such as are induced by the administration of excessive amounts of these hormones to normal animals seldom, if ever, occur.

Swingle and associates (137) have observed that adrenalectomized dogs may develop edema and circulatory collapse following transfusions of blood serum but that normal dogs fail to react in this manner. The sensitivity of the adrenalectomized dog to homogeneous transfusions of serum is unexplained.

The reason for the increased requirements for the cortical hormones during stress is unknown. Is it due to increased catabolism or excretion of these hormones or are larger amounts involved in physiologic processes?

Other physiologic processes. Gaunt and Tobin (138) reported that lactation could be partially maintained in adrenalectomized rats given an optimal intake of sodium chloride. The compound 11-desoxycorticosterone would be expected to support lactation as well as does sodium chloride for it maintains a positive sodium balance in the body but Gaunt (139, 140) found it not only failed to support lactation but in some cases actually suppressed it. Other cortical steroids were found capable of sustaining lactation in a nearly normal manner. Spoor et al. (44) described a fraction from adrenal cortex extracts which maintained lactation in adrenalectomized animals and stimulated the crop gland of the pigeon. This work was repeated by Hurst, Meites and Turner (141) with negative results. The relationship of the cortical hormones to lactation and to the processes supporting it is not clear.

Effects of the cortical principles on the nervous system have been described (142, 143, 144). Do the cortical principles have effects which are specific to nerve tissue, or are nerve changes secondary to specific effects remote from nerve tissue or does the nervous system share in some general tissue effect of these hormones?

The nature of the asthenia of cortical insufficiency is not known. Ingle and Lukens (145) found circulatory and carbohydrate deficiencies to be factors in the 'fatigue' of adrenalectomized rats under experimental conditions that were atypical and may have little in common with the conditions of chronic insufficiency. The question as to whether there is some fundamental defect in the metabolism of muscle contraction other than those due to lowered carbohydrate stores and circulatory collapse has not been answered.

Following adrenalectomy the thymus of the rat may increase in size. The adrenal steroids share with the sex hormones the property of causing atrophy of the thymus and lymph nodes of the rat. The mechanism is unexplained.

Ingle failed to maintain a normal work capacity in adrenalectomized rats by treatment either with whole extract (146) or with a mixture of 11-desoxycorticosterone and 17-hydroxy-11-dehydrocorticosterone (64). Is this deficiency due to some qualitative deficiency in the replacement therapy used or is it due to failure to duplicate the physiologic conditions of hormone secretion?

The mechanism of pigmentation during cortical insufficiency in the human being and the reason for its failure to disappear during treatment is still obscure.

COMMENT

The study of any fundamental problem in science is likely to raise more new questions than are answered by the investigation. In the field of investigation of the adrenal cortex the relatively few general questions recognized prior to the isolation of active principles have, during the past 12 years, been replaced by a bewildering number of more specific questions regarding its control and function. It may be anticipated that in the light of future advances many of the questions formulated today will give way to more penetrating inquiry into the ultimate mechanisms of cortical hormone metabolism and activity.

Research in the field of investigating the adrenal cortex has been dominated by the need to isolate those principles which are essential for life and for an analysis of the physiologic deficiencies which lead to the death of the untreated animal or patient. The isolation and chemical identification of life-maintenance principles has been accomplished and their property of maintaining life under optimal conditions has been definitely shown to be concerned with the proper distribution of water and electrolytes in the organism. Now the chemist, physiologist and the clinician can work toward the broader objective, that of becoming able to restore to the animal or patient complete normality in all phases of metabolism and adaptability to stress which characterizes organisms without disease.

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RÔLE OF ADRENALS IN PRODUCTION OF TRAUMATIC SHOCK IN RATS[1]

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THE NORMAL RAT appears to be resistant to the production of shock following severe muscle trauma. Although the mechanism of this reaction is obscure, resistance to this type of shock seems to be dependent upon the presence of the adrenal glands since muscle trauma in adrenalectomized rats readily produces shock and death (1).

The present studies were undertaken to obtain information concerning the following questions which arise. *a*). What is the quantitative degree of the normal rat's resistance to traumatic shock as compared to that of other species? *b*). What mechanisms are utilized by normal rats to cope with sublethal injury? *c*). Why does trauma produce shock and death in adrenalectomized rats?

METHODS AND RESULTS

Effect of Trauma upon Normal and Completely Adrenalectomized Rats

The rats used in this study, weighing between 80 and 120 gm., were fed Purina dog chow biscuits. The bilaterally adrenalectomized rats, operated 5 to 7 days prior to the date of experimentation, were maintained in excellent health post-operatively by substituting saline for drinking water and by feeding them bread as well as Purina biscuits *ad libitum*.

Trauma was administered to etherized rats by beating one or both hind legs with heavy rubber suction tubing. The duration of anesthesia in every case was approximately 10 minutes during traumatization. The following standardized procedure for damaging the hind leg was used. The paw was struck 100 forceful blows after which 200 blows were administered to the extensor surface of the limb. The degree of injury, as evaluated by immediate edema, seems to be maximal since striking the paw 200 times or the extensor surface 300 to 400 times produces no greater immediate loss of fluid into the injured area. The traumatic procedure employed regularly breaks the fibula and irregularly produces a compound fracture of the tibia. In the latter case, however, the loss of blood through the broken skin is negligible. Examination of a damaged hind limb reveals marked hemorrhagic areas in the subcutaneous tissues, muscle fascia, especially in the intermuscular spaces and to a smaller degree in the muscles.

Semi-quantitative evaluation of the fluid lost into the injured area was accomplished by weighing the normal and damaged lower extremities of rats obtained in the following manner. After the viscera had been removed and the tail cut off, a midline incision was made on the back extending from the anus to the base of the neck. The lower portion of the body was divided from the upper half by cutting through all of the tissue layers and the spinal column on a line parallel to, and just below the lowest ribs of the thoracic cage. Paravertebral incisions, completely isolating the verte-

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bral column, were used to separate the two lower extremities. Using this method, it has been found that the edema, immediately evident after cessation of beatings, amounts to 2.5 to 4.0 per cent of the body weight when one leg is damaged, and to 4.5 to 6.0 per cent of the body weight when both legs are damaged. Fluid loss into the area of injury was of the same order of magnitude in the group of adrenalectomized rats and the normal group, no significant differences being noted between these animals.

Table 1 summarizes the findings obtained by traumatizing one or both hind limbs of normal and completely adrenalectomized rats. It reveals that all of the adrenalectomized rats died following injury to one or both legs, while normal rats regularly survived without mishap. The fate of the animal following trauma depended entirely upon whether the rat was normal or adrenalectomized, and was not influenced either by sex or the production of compound fracture. Emerging from anesthesia,the injured

TABLE 1. EFFECT OF STANDARDIZED TRAUMA TO THE HIND LEGS UPON THE FATE OF NORMAL AND COMPLETELY ADRENALECTOMIZED RATS

Type	No. of Rats	No. of Legs Injured	No. Survived	No. Died in Shock	Av. Time of Death, Hrs.	Range
Normal rats	18	1	18	0		
	20	2	20	0		
Bilaterally adrenalectomized rats	25	1	0	25	6.0	3.9–9.0
	20	2	0	20	3.0	0.7–6.0

adrenalectomized rat passed through two distinct phases before dying. The following description may serve to characterize these. *Phase 1*. The rat is hunched up, the eyelids are closed, the luster of the eye appears dulled, respiration is irregular and, later, the animal becomes cold. *Phase 2*. The rat exhibits profound asthenia, appears almost comatose and eventually dies. No attempt will be made to designate either *phase 1* or *2* as shock in view of the absence of a generally accepted definition for this syndrome. The sole criterion for traumatic shock, in this study, is death which follows *phase 1* and *2*. In marked contrast to adrenalectomized rats, normal animals following injury do not show the symptoms described in *phases 1* and *2*.

The finding that normal rats withstand severe trauma to both hind legs, suggests but does not prove, that the rat is more resistant to trauma than other species are, since dogs and cats and man may develop shock after traumatization of only one limb. However, in view of the difficulties involved in the quantitative evaluation of damage produced by beatings, comparative estimation of species' resistance on this basis is not very satisfactory. On the other hand, injury produced by temporary obstruction of the blood flow appears to be a shock-producing procedure, wherein comparable results between rats and other species might be more easily obtained. In this type of tissue damage, the factors involved may be listed as *a*), the duration of obstruction of blood flow *b*), the degree of occlusion of blood flow and *c*), the amount of tissue rendered ischemic. It will be seen that these factors can be adequately controlled. Selye and Dosne (2) have reported that obstruction of the blood flow to a hind limb of a monkey for 6 hours (produced by placing a rubber tourniquet around the upper part of the thigh) led to death from shock in 3 of 6 animals following release of blood flow. In this laboratory (6 experiments) similar obstruction of the blood flow to a single hind limb of rabbits produced shock and death in all cases upon release of the rubber tourniquet. Using this technique for producing tissue injury, experiments were undertaken to obtain data regarding the comparative resistance of rats to temporary oc-

clusion of the blood flow. Rubber tourniquets were placed tightly around the upper part of the thighs of both hind legs for variable periods of time and subsequently released. Table 2 illustrates the results obtained in normal and completely adrenalectomized rats subjected to this procedure. It will be seen that occlusion of blood flow to both hind limbs for 6 hours in normal rats does not cause death, in marked contrast to the findings obtained in rabbits and reported in monkeys. Doubly adrenalectomized rats, however, are killed by temporary obstruction of blood flow.

Comment. The results presented demonstrate that normal rats possess a quantitatively higher capacity for resisting shock and death than rabbits and monkeys. It is of especial interest that the traumatized normal rat does not require exogenous treatment with fluids for survival. Presumably, if man possessed the resistance exhibited by rats, shock would be much less of a problem than it now represents. Investigation of the quantitative rate of fluid loss into a damaged area revealed no differences between

TABLE 2. EFFECT OF TEMPORARY OBSTRUCTION OF BLOOD FLOW IN NORMAL RATS AND RABBITS AND IN ADRENALECTOMIZED RATS

Type	No. of Animals	No. of Legs Occluded	Hours of Blood Flow Occlusion	No. Survived	No. Died in Shock
Normal rats	10	1	6	10	0
	10	2	6	10	0
Normal rabbits	6	1	6	0	6
Bilaterally adrenalectomized rats	11	1	6	2	9

normal and adrenalectomized rats. It is therefore apparent, as Freed (1) has previously pointed out, that the production of shock and death in traumatized adrenalectomized rats and the survival of injured normal rats which lose as much or more fluid into the area of injury, is evidence against Blalock's theory of local fluid loss (3).

Effect of Unilateral Adrenalectomy upon the Resistance of the Rat to Trauma

The high resistance of the rat to traumatic insults, of intensity sufficient to induce shock and death in other experimental animals, is dependent upon the presence of the adrenal glands. The possibility arises that the rat possesses a quantitatively higher degree of adrenal activity than man or other animals. Removal of one adrenal gland in the rat might possibly be expected to reduce the total adrenal functional capacity to levels comparable to those found in other species. Reduction of adrenal functional capacity, on this basis, would be followed by reduction of resistance of the rat to trauma. To test this idea, the effect of standardized trauma upon unilaterally adrenalectomized rats was studied. Fifteen rats, unilaterally adrenalectomized 3 to 5 days prior to the date of the experiment, were struck 300 blows on each leg in the usual manner, and their fate compared to similarly traumatized sham adrenalectomized rats. Table 3 illustrates the results obtained in this experiment. It will be seen that 11 of the 15 unilaterally adrenalectomized rats died in shock after trauma, while 4 survived injury. The unilaterally adrenalectomized rats which survived exhibited the symptoms described previously in *phase* 1, except that they never became cold. Those rats which survived did not pass into *phase* 2, they subsequently recovered and appeared normal except that marked edema was evident in both damaged limbs. In marked contrast to unilaterally adrenalectomized animals, normal rats subjected to sham adrenalectomy suffer no lowering of resistance to trauma. These results obtained in rats uni-

laterally adrenalectomized for 3 to 5 days, demonstrate that reduction of adrenal functional capacity markedly reduces the resistance of the rat to injury. Preliminary experiments in this laboratory indicate that rats weighing 80 to 120 gm. 3 to 4 weeks after unilateral adrenalectomy regain resistance to standardized trauma. Furthermore, old rats weighing between 200 to 280 gm., unilaterally adrenalectomized for 3 to 5 days, while less resistant to standardized beatings than normal rats, are significantly more resistant than young unilaterally adrenalectomized rats weighing 80 to 120 gm.

Comment. There is one important difference between completely and unilaterally adrenalectomized rats which warrants comment. While all of the bilaterally adrenalectomized animals died, 4 of 15 unilaterally adrenalectomized rats survived. It has long been recognized that the procedures employed to produce experimental shock in dogs and cats, whether by hemorrhage, crushing of muscles, infliction of burns, exposure

TABLE 3. EFFECT OF UNILATERAL ADRENALECTOMY UPON RESISTANCE OF RATS TO TRAUMA

Type	Days After Operation	No. of Rats	No. of Legs Injured	No. Survived	No. Died in Shock	Time of Death, Hrs.
Unilaterally adrenalectomized rats	3–5	15	2	4	11	0.5–48[1]
Sham-adrenalectomized rats	3–5	10	2	10	0	

[1] These animals died at 0.5, 0.75, 0.8, 1.0, 2.0, 2.0, 2.5, 3.0, 36–48, 36–48, and 48 hours, respectively, after trauma.

and manipulation of the gut while all capable of producing shock, do not invariably do so (4). This irregular response to standardized trauma of the hind legs is likewise shown by unilaterally adrenalectomized rats but not by completely adrenalectomized or normal animals. In this respect then, unilaterally adrenalectomized rats respond to trauma as do normal cats, dogs and monkeys. From our data it appears that the resistance of the rat to trauma of the hind legs is quantitatively, and not qualitatively, related to the activity of the adrenal glands. If one assumes that resistance to shock in other species is similarly related to the functional activity of the adrenal glands, then it may be possible to explain the variable response to shock-producing procedures on the basis of quantitative differences in adrenal activity between animals. The suggestion is advanced that resistant dogs and cats may possess a quantitatively higher degree of adrenal activity than sensitive animals.

CAPILLARY PERMEABILITY CHANGES INDUCED BY TRAUMA IN NORMAL AND COMPLETELY ADRENALECTOMIZED RATS

Although there are marked differences of opinion with regard to the initiating mechanisms of shock, there is considerable agreement in that a vicious cycle of fluid loss from the vascular system operates as an important contributing mechanism to the development of shock (3, 5). This concept may be summarized briefly as follows. Reduction in blood volume (produced either by a toxin or by fluid loss as a result of trauma)→reduced blood flow→tissue anoxia (including lung)→increase in capillary permeability→further loss of fluid and reduction of blood volume. The possibility arises that the protective activity of the adrenal glands of the normal rat operates through interruption of this vicious cycle at some point so that generalized increase of capillary permeability is prevented. Investigations were, therefore, undertaken to ascertain *a*), the effect of shock upon generalized capillary permeability in traumatized adrenalectomized rats and *b*), the effect of trauma upon generalized capillary permeability in shock resistant normal rats.

Capillary permeability is a term with little meaning unless it is related to the pas-

sage of a specific substance or group of substances through the capillary wall, in view of the fact that the capillary membrane is permeable to some molecules and relatively impermeable to others. Ions, small molecules, and water readily diffuse through capillaries so that the concentrations of these substances in plasma and tissue fluid are practically equivalent. On the other hand, plasma proteins and other large molecules are relatively impermeable to most capillary membranes (8). In considering the effects of alterations of capillary permeability to particular substances upon fluid exchange between plasma and tissue fluid, it is apparent that increase of capillary permeability to ions would have negligible effect on fluid exchange, since permeability to ions is already complete. On the other hand, increase in the permeability of capillaries to plasma protein would produce serious consequences to fluid exchange, for in this instance plasma protein would pass through the capillary wall at an increased rate to enter the extracellular fluid compartment. The presence of protein on both sides of a capillary membrane which is no longer impermeable to large molecules would upset the osmotic relationships between plasma and extracellular fluid, and would relatively increase the capillary filtration pressure, so that excessive accumulation of tissue fluid would result. It is clear, therefore, that in considering fluid loss from the vascular system, alteration of capillary permeability is important only so far as it affects the permeability of large colloidal-sized particles such as protein. The term capillary permeability when used in this paper is, therefore, defined as the passage of large-sized particles through capillary membranes. To study changes in capillary permeability, the passage of a colloidal dye stuff, trypan blue, through capillaries has been utilized. Unpublished observations which will be reported subsequently indicate that the dye combines with plasma protein, and therefore may be an actual index of protein passage through capillary walls.

The method employed for evaluating changes in generalized capillary permeability has been previously described in full detail (6) The basis of the method may be summarized briefly as follows. Following the intravenous administration of a colloidal dye stuff, trypan blue, the dye circulates, and passes through those capillary membranes of sufficient porosity and stains the tissue. After the intravenous injection of an arbitrarily chosen dose of trypan blue, which produces a concentration of dye in tissue intermediate between minimal and maximal staining, the animal is killed. The effect of the stimulus under investigation upon capillary permeability is obtained by comparing the degree of dye concentration in the tissues of a group of treated rats with those in the tissues obtained from untreated control rats. This was done under conditions whereby rats of both groups received equivalent amounts of dye which was allowed to circulate for the same time interval. The quantity of dye within a stained tissue depends upon the amount of dye within the vascular channels and within the tissue proper. The latter factor depends upon the balance between the rate of dye penetration and the rate of dye removal which is, in turn, influenced by the degree of dye fixation. By correcting for the effects of extra-capillary permeability factors upon dye accumulation, the effect of the stimulus upon capillary porosity is indirectly obtained. It must be emphasized that the method described is at best only semi-quantitative and is capable of detecting only large changes in capillary permeability.

Effect of Trauma upon Generalized Capillary Permeability in Normal and Completely Adrenalectomized Rats (Excluding the Local Area of Injury)

The effect of shock upon generalized capillary permeability was indirectly evaluated in traumatized, completely adrenalectomized rats, during both the initial and later stages of shock, using the methods described above. Simultaneous experiments were performed with traumatized normal rats and with control uninjured adrenalec-

tomized and normal rats, so that the effect of trauma alone and of adrenalectomy alone on capillary permeability could be simultaneously analyzed and corrected for. Eight rats were used in each group. All rats received 1.0 cc. per 100 gm. of body weight of a 1 per cent trypan blue solution in saline intravenously through the tail vein while anesthetized with ether. They were killed with ether approximately 30 minutes after dye injection. A few minutes after the trypan blue injection, one group of 8 completely adrenalectomized rats, while still under ether, were traumatized on one leg in the usual manner. Eight normal rats were traumatized in similar fashion after dye had been intravenously administered. With the exception of the traumatized leg the findings on macroscopic examination were as follows.

Effect of adrenalectomy on generalized dye accumulation. Comparing the degree of dye accumulation in the tissues of non-traumatized normal and adrenalectomized rats, it was observed that following adrenalectomy a slight but definite increase of dye concentration was observed in lung tissue and possibly in certain areas of the gastrointestinal tract, the ear, paw and tongue. Adrenalectomy is without significant effect on capillary permeability in other tissues such as skin, kidney, muscle, brain, heart, liver, spleen and gonads. The findings in the lungs are not necessarily the result of adrenalectomy, *per se*, since it is possible that adrenalectomized rats are more sensitive to the irritative action of ether upon the respiratory tract than are normal rats.

Effect of trauma not resulting in shock upon generalized dye accumulation. The degree of dye accumulation in the tissues of traumatized and control normal rats, excluding the traumatized area, revealed no significant differences between these two groups.

Changes in dye accumulation during the initiation of shock (excluding the area of injury). The degree of dye accumulation in the tissues of traumatized adrenalectomized rats and of control uninjured adrenalectomized rats were compared. No significant differences were found in these groups except that in the traumatized adrenalectomized rats considerably larger amounts of the dye were concentrated in the lungs than in the non-injured adrenalectomized rats. Comparison of traumatized adrenalectomized tissues with those of traumatized normal rats revealed no differences that could not be accounted for on the basis of adrenalectomy alone, with the exception of the finding in the lungs. It thus appears that dye accumulation in the pulmonary tissues is increased following trauma in etherized adrenalectomized rats. However, this increased accumulation observed in the lungs of etherized adrenalectomized rats is not crucial evidence that trauma necessarily increases pulmonary capillary permeability. It must be remembered that the duration of etherization in our traumatized adrenalectomized rats was 10 to 12 minutes longer than in the control uninjured adrenalectomized animals. Assuming that adrenalectomized rats are highly sensitive to the respiratory irritation produced by ether, it is possible to explain our finding on the basis that the increased duration of etherization, and not trauma, is the causative factor in the production of increased dye accumulation observed in the lungs of injured adrenalectomized animals. Experiments described in the following section evaluate this possible ether action upon capillary permeability.

Changes in dye accumulation in later stages of shock. It has been claimed that in the later stages of shock, generalized capillary permeability is irreversibly increased so that the vascular system can no longer retain colloidal-sized particles (5). The next investigations were undertaken to obtain direct evidence on this point. Six completely adrenalectomized rats were traumatized on both hind legs in the usual way. When the rats were clearly approaching *phase 2* (the rats were cold, but not profoundly asthenic) they were etherized and trypan blue was injected intravenously. If still alive, the adrenalectomized rats in shock were killed 30 minutes later. Two of the 6 rats died 20 and 25 minutes after dye administration. In some of these animals it was necessary to

inject the trypan blue solution into the exposed jugular vein since it was not possible to inject the tail vein. Comparison of the degree of dye accumulation in the tissues of rats in the later stages of shock with those obtained during the initiation of shock, revealed no significant differences between the two groups. These results indicate that in the initiation and in the later stages of shock in the etherized rat, there is no generalized increase of accumulation of trypan blue in the tissues, except in the lungs. To test the possibility that the increased dye accumulation observed in the lungs of traumatized adrenalectomized rats might be due to the irritation of the ether used for dye injection, further experiments were performed. *a*), Dye accumulation in the tissues of adrenalectomized rats in shock was studied under conditions in which trypan blue was administered in the absence of ether. *b*), The weight of lungs of animals dying in shock were compared to those obtained from normal animals killed for purposes of comparison. Eight completely adrenalectomized rats were traumatized on both legs; when they were at the end of *phase 1*, obviously sick, trypan blue was administered intravenously through the tail vein without ether anesthesia. Thirty minutes later, these rats were killed by a blow on the head, and dye accumulation in the tissues studied. In contrast to those animals which had received dye under ether anesthesia, 7 of the 8 animals injected without etherization did not show increased dye accumulation in the lungs. Examination of the other tissues revealed no significant differences from tissues of uninjured adrenalectomized rat. It therefore appears that the increased dye accumulation observed in the lungs of traumatized adrenalectomized rats which received dye during etherization, is due to the ether and is not produced by trauma. To check this conclusion, the lungs of rats which died in shock were weighed and compared to the weight of lungs of animals killed by a blow on the head. Table 4 illustrates this data and demonstrates that the rats dying in shock did not accumulate excess fluid in the lungs which presumably would result if capillary permeability were increased in this tissue. It may be mentioned that a high degree of pulmonary congestion is evident in some rats subjected to shock. However, this congestion was not consistently observed, many rats dying in shock having normal-appearing lungs.

TABLE 4. COMPARISON OF LUNG WEIGHTS OF RATS DYING IN SHOCK WITH THOSE FROM RATS KILLED BY A BLOW ON THE HEAD

Type of Death	Group	No. of Rats	Av. Lung Weights, gm/100 gm. of Body Weight	Range
Shock	Bilateral adrenalectomy	10	0.83	0.70–1.10
	Unilateral adrenalectomy	10	0.85	0.78–1.03
Blow on head	Unilateral adrenalectomy	8	0.85	0.77–1.10
	Normal	8	0.88	0.80–1.20

Comment. The evidence presented in the previous sections indicates that a generalized increase of trypan blue accumulation is not associated either with the initial or later stages of traumatic shock in the rat. Although the traumatized adrenalectomized rat in shock does show increased dye accumulation in the lungs when trypan blue is administered to etherized animals, this finding appears to be due to pulmonary irritation produced by ether, since dye accumulation in the lungs is normal when dye is administered to non-etherized traumatized adrenalectomized rats.

Before it is possible to evaluate the effect of shock upon capillary permeability to trypan blue from the data on dye accumulation in tissues, extra-capillary factors which

may affect permeability and dye accumulation must be taken into account. The first factor, that of the degree of dye fixation, was studied in vitro using the method described in a previous paper (6) wherein the degree of dye absorption by various tissues is semi-quantitatively evaluated. No significant differences between normal, adrenalectomized, traumatized normal or traumatized adrenalectomized tissues were observed. Thus possible differences in dye adsorption which might be expected by the induction of shock, do not appear to be important in influencing dye accumulation. If shock is associated with an impaired blood flow to tissues (in our experiments there is good evidence for this in the skin, since our animals are cold) the amount of intravascular dye would tend to be reduced, which in turn might decrease tissue coloration. However, the effect of reduced blood flow on the amount of intravascular blue can be shown to have slight importance for tissue coloration. If the intravascular content of dye is raised in normal animals by increasing the dosage of intravenous dye four-fold, in those tissues with non-porous capillaries for example, brain and gastrocnemius muscle, the coloration is increased only little if at all (6). It therefore appears that differences in the intravascular content of dye which may be present in shock are without serious influence upon tissue coloration. The effect of impairment of blood flow upon dye accumulation in tissues where capillary permeability is normal must next be considered. In all normal tissues, with the exception of skin, dye accumulation (for the dose of administered dye) is maximal after the dye has circulated for one minute following injection, and either remains constant or decreases (6). This finding indicates that the rate of passage of trypan blue through normally porous capillaries is fast and occurs maximally within approximately one minute. In a tissue with reduced blood flow, the number of dye molecules which reach the capillary filtration surface per unit of time is decreased. If the blood flow is halved, for example, then there will be half of the normal number of molecules of trypan blue reaching the filtration surface at any one time and it will require twice as long for the same number of dye molecules as in the normal case to come in contact with the capillary wall Since there is an upper limit for the number of dye molecules which are filtered and fixed, it is clear that other things being equal, a tissue with reduced blood flow and normal capillary porosity will, in sufficient time, accumulate amounts of dye equivalent to that of normal tissues. Since the time interval necessary to allow maximal dye accumulation in all tissues except skin is in the range of one minute, and taking into consideration the fact that in our experiments dye was allowed to circulate for 30 minutes, it would appear that the blood flow to organs could be reduced many fold, possibly to the neighborhood of 30 times, without influencing dye accumulation. In the case of skin where dye accumulation does increase with time during the 30 minutes following dye injection, this reasoning does not obtain and it will, therefore, be necessary to discuss this tissue separately.

The data on dye accumulation in shock, excluding skin and the area of injury at the moment, is apparently not seriously influenced by factors such as dye fixation, the concentration of intravascular dye, and moderate changes in the rate of blood flow to tissues. It therefore appears that the only other factor which influences dye accumulation in these tissues is capillary porosity. Since dye accumulation in the undamaged tissues was normal, it appears that the capillary porosity of all uninjured tissues (excluding skin) was not influenced by shock. This appears to be the case not only during the initiation of shock, but also during the later stages, when the rat is close to death. It may be mentioned that this finding presents evidence against the idea that a toxic substance which acts by producing generalized increase of capillary permeability is necessarily operative in the production of traumatic shock.

The dye accumulation observed in skin following dye administration is initially slight and increases continuously at a slow rate so that after 24 hours, the skin is deeply blue. This indicates that the number of dye molecules which are fixed in skin per unit of time is small, and that during the first 30 minutes following injection of dye, the maximal degree of tissue staining is not obtained. In skin, therefore, reduction in blood flow would reduce dye accumulation. However, if capillary permeability in skin is significantly increased (by xylol for example), the amount of dye concentrated within minutes is equivalent to that amount found after many hours in normal skin. This finding indicates that with increased capillary porosity, skin requires only a short time to achieve maximal staining. It would appear that, even if the blood flow were reduced considerably in skin with permeable capillaries, dye accumulation within 30 minutes should be considerable and much greater than that observed in normal skin. The finding that dye accumulation in skin of rats in shock is the same as that in animals not in shock, while indicating an actual increase of capillary permeability (since blood flow appears to be decreased) suggests that any such change was not of large magnitude. When it is realized that the method employed in these studies is at best only semi-quantitative and that only large differences in tissue coloration can be safely evaluated, it is apparent that slight differences cannot be measured. On this basis, the results obtained in skin with our rats in shock may indicate that capillary permeability was little if at all increased in shock.

Effect of Trauma upon Capillary Permeability in the Localized Area of Injury in Normal and Adrenalectomized Rats

The changes in dye accumulation of the localized area of injury in traumatized normal and adrenalectomized rats were similar, thus no distinction will be made in this section between these two groups of animals. Rats (10 normal, 8 adrenalectomized) were injected with trypan blue intravenously and 3 to 5 minutes later, one leg of each animal was traumatized in the usual manner. Thirty minutes later the animals were killed with ether and the legs examined, the non-traumatized limb serving as a control for comparison. Marked dye accumulation occurred in the traumatized skin, subcutaneous tissue, muscle fascia, and in the muscle spaces where large amounts of trypan blue were evident along with blood. A slight increase in dye accumulation was observed in the traumatized muscle. Since injury to skin and muscle was without effect on the degree of dye fixation determined in vitro, these results indicate that following trauma to the hind leg the capillary porosity in skin, subcutaneous tissue, muscle fascia, and to a lesser degree in muscle, was increased. This finding may be due to capillary rupture, since large amounts of blood also escape into the tissues. Whether there is increased capillary permeability or actual breakdown of the capillary wall, large molecules such as protein would presumably escape from the vascular system into the tissues. However, if rats were traumatized first and then dye injected at various time intervals thereafter, an entirely different picture of dye accumulation was obtained in the traumatized leg. The damaged limbs of rats traumatized 10 to 60 minutes before dye injection, showed no significant accumulation of dye either in skin, subcutaneous tissues, or muscle. Hemorrhage into the muscle spaces and the subcutaneous tissues was nevertheless evident, although no significant amounts of blue dye were found associated with these hemorrhagic edematous areas. It is of interest to note that the slight coloration of skin produced by dye in the normal limb was absent in the damaged limb, indicating a marked deficiency in blood flow through the area of injury.

These results demonstrate that the increased capillary permeability produced

locally in a traumatized limb in both normal and adrenalectomized rats, is of a short and transient duration and is abolished within 10 minutes. Investigation of the mechanism of reversal of the increased dye accumulation produced by trauma (using hind paws in these studies instead of the whole limb) led to the following findings. *a*), The time of reversal is increased from 10 minutes to 2 or 3 hours if trauma is administered in the presence of an obstruction to blood flow, the tourniquet being allowed to remain in place 10 minutes after injury (6 normal, 6 adrenalectomized animals). *b*), Trauma, followed in a few minutes by application of a rubber tourniquet allowed to remain in place for 10 minutes, was followed by immediate reversal of the increased dye accumulation (12 rats, 8 normal, 4 adrenalectomized). The effect of obstruction of blood flow *per se*, does not influence the rate of reversal, since following trauma this factor is without effect. The finding that obstruction of blood flow before trauma markedly decreases the rate of reversal indicates that the rapid reversal effect is dependent upon blood flow during the period of injury. The suggestion is advanced that the rapid reversal of the increased dye accumulation by trauma may be due to mechanical blockade of injured capillaries, possibly with fibrin and blood cells with a consequent shift of flow to less porous capillary channels. The previously mentioned finding that the injured leg does not have the normal amount of coloration indicates that the blood flow through non-injured capillaries is less than normal. That some capillaries are open and carrying blood is evident, however, from the facts that the degree of edema increases with time, and that gangrene does not develop in the damaged limbs of normal rats. The marked decrease in the rate of reversal produced by obstruction may be explained on the basis that blockade of injured capillaries does not initially ensue in the absence of blood flow. Thus when circulation is re-established, trypan blue is lost through the damaged capillary walls. However, blockade is instituted at a slow rate, so that, in time, the injured capillaries are blocked and blood flow is shifted to less porous capillary vessels.

Comment. The results presented in this section indicate that trauma produces a transient increase of capillary permeability in the area of injury, possibly by rupture of capillaries. The reversal of increased capillary porosity is presumably related to mechanical blockade of the damaged capillaries with fibrin and cells. If these conclusions are valid, it is necessary to explain progressive fluid loss into an injured area in the face of normal capillary permeability relationships. Bayliss (7) has previously explained how such a phenomenon could occur on the basis of Starling's theory of fluid exchange. Injury is initially associated with loss of the colloidal-sized blood components into the tissue fluid of the damaged limb. Although increased capillary porosity is subsequently reversed, plasma protein is now present in the tissue fluid where its presence upsets the normal forces operating to prevent excessive fluid loss from the capillaries. The osmotic pressure exerted by the plasma proteins in the non-injured capillaries, which tends to hold fluid in the capillaries, is partially or completely (depending on concentration differences) negated by the presence of tissue protein on the other side of the capillary membrane. Lowering of the osmotic pressure of plasma likewise increases the relative capillary filtration pressure which thus drives fluid through the capillary wall at increased rate. It seems almost necessary to assume that a slow rate of lymphatic removal of protein must be associated with trauma so that the tissue fluid protein is not rapidly diminished. This may be due either *a*), to the inactivity of the damaged limb, inactivity decreases lymph flow (8) *b*), to mechanical blockade of lymphatic vessels with fibrin or *c*), to both factors operating simultaneously. It will be thus seen that with normal capillary permeability, damaged tissue can progressively become more edematous.

Effect of Asphyxia upon Capillary Permeability

The results presented in the previous section indicate that there is no evidence for the assumption that there is a generalized increase in capillary permeability, except possibly in skin, either in the initial or later stages of shock in the rat. Similarly, no evidence has been obtained which supports the concept that a vicious cycle in which anoxia, produced through reduction in blood flow, increases capillary permeability. In view of the fact that anoxia is generally believed to produce increased capillary permeability, experiments were undertaken to re-investigate this point.

The principal evidence for the theory that anoxia increases capillary permeability may be briefly summarized as follows. *a*), Capillaries of the frog mesentery bathed with O_2-free Ringer's solution become abnormally permeable when they are obstructed for 0.5 to 3 minutes (9). *b*), Perfusion of the frog web with 3 per cent acacia leads to edema in the absence of ox red cells (10). *c*), Anoxemia in dogs is accompanied by an increase in lymph flow and by an increased content of lymphatic protein (11, 12). It may be pointed out, however, that *a* cannot be demonstrated from the O_2-lack produced by obstruction of blood flow alone, but requires in addition a complete O_2-free environment. The drastic reduction of O_2 obtained in this experimental situation hardly appears comparable to the degree of anoxemia observed in shock. The significance of *b* for the demonstration of the rôle of oxygen upon capillary permeability is diminished by findings that carbon particles (13) or platelets (14) may replace red cells in the prevention of edema. The evidence presented in *c* does not give information regarding changes in capillary permeability produced by anoxia in the various tissues of the body. As against this evidence, the concept that oxygen-lack may not increase capillary permeability in man, has been suggested by McMichael and Morris (15) who observed that the rate of swelling produced by a sphygmomanometer in man is not altered when the inspired air is low in O_2 or high in CO_2.

The effect of generalized asphyxia upon capillary permeability in normal rats was obtained by comparing the degree of dye accumulation in the various tissues of rats killed by asphyxia in the presence of circulating dye, with that in control rats killed by a blow on the head. Normal rats, while anesthetized with avertin, were injected via the tail vein with trypan blue. Immediately, thereafter the rat was placed in a Bell jar and the top sealed. A control rat was injected with an equivalent amount of dye, left in the air, and killed by a blow on the head after the dye had circulated for an equivalent time interval. Variations in the time interval required to produce death by asphyxiation on this basis was accomplished by using jars of various volume and by changing the volume with inert material. In all cases, xylol injected intradermally into one hind limb of the control non-asphyxiated rats, 5 minutes before they were killed by the blow on the head, produced definite dye accumulation in the injected area. This finding, therefore, demonstrates that during the periods of asphyxia, adequate amounts of trypan blue were circulating to produce dye accumulation in response to an adequate stimulus. Examination of the tissues of 7 animals dying from generalized asphyxia revealed no significant differences in dye accumulation from the control tissues. The rats died within the jar after 15, 25, 30, 40, 50, 60, and 110 minutes, respectively. It is, therefore, apparent that generalized progressive asphyxia sufficient to produce death in from 15 to 110 minutes does not affect tissue capillary permeability to trypan blue. However, the significance of this finding is limited by an objection that the generalized asphyxia might produce death without necessarily producing local oxygen deficiency sufficient to affect capillary permeability.

Experimental conditions were thus designed to overcome this objection and to allow evaluation of the effect of asphyxia on capillary permeability in two specific

tissues, skin and muscle. Under avertin anesthesia the left common iliac artery and vein of normal rats were exposed. After periods of asphyxia (produced by clamping both the left common iliac artery and vein) of 1, 3, 5, 10, 20, 25, 30, 45, and 60 minutes duration, trypan blue was injected intravenously and the clamp was removed 1 to 3 minutes later. In other experiments, after dye had been injected, the iliac artery and vein were clamped for 1, 3, 5, and 10 minutes, after which time the clamp was released. At least 3 rats were used at each time interval. The skin was observed directly throughout the experimental period and muscle was examined 30 to 60 minutes after the clamp had been released. The effect of asphyxia on the permeability of the capillaries of the skin and muscle were evaluated by comparing the degree of dye accumulation in the asphyxiated leg with that of the normal control limb. In no instance was there any difference between the two legs either in skin or muscle, regardless of the duration of asphyxia or whether the dye had been injected before or after obstruction of the circulation. It was apparent from the following facts that the circulation to the leg was occluded by clamping the common iliac vessels. *a*), The skin temperature, as measured by a McKesson Dermalor, rapidly fell upon obstruction below that of the control limb. *b*), Following the intravenous injection of dye a slight bluish coloration was evident in abdominal skin and in the skin of the control limb, but not in the clamped-off leg. *c*), Cyanosis rapidly appeared in the obstructed leg. The circulation in the obstructed leg was adequately re-established when the clamp was removed as evidenced by the following findings. *a*), The skin temperature of the obstructed leg rapidly increased to that of the normal limb if the duration of asphyxia was less than 45 minutes. Following asphyxia of 45 and 60 minutes, release of the clamp caused a rapid rise in skin temperature, although not to the level of the temperature of the control skin, indicating that blood flow following obstruction for these intervals is somewhat below normal; *b*), the slight coloration of skin, produced by intravenous injection of dye, absent from the occuluded limb, upon release of the clamp, appears in the obstructed leg so that within a few minutes the two legs have the same degree of coloration; *c*), the injection of xylol into the foot of the occluded leg produces a marked local dye accumulation upon release of the clamp. The rate of dye accumulation produced by xylol in the control and asphyxiated limb after circulation has been re-established was likewise the same.

That the capillaries of skin and muscle do accumulate trypan blue when presented with an adequate stimulus to increased capillary permeability is evident from the finding that xylol causes dye accumulation in skin; and that trauma induced by repeated beatings causes due accumulation in muscle. The demonstration that periods of asphyxia for one hour or less are without effect on capillary permeability in skin and muscle, must, therefore, be taken to indicate that asphyxia of these durations is an inadequate stimulus.

To check the effect of asphyxia upon the permeability of capillaries in rat skin, obstruction of blood flow was produced by rubber band tourniquets which were tightly applied around the thighs of normal rats for varying intervals of time. Obstruction by the rubber band tourniquet was at least as effective in skin as that produced by clamping off the common iliac vessels as judged by fall in skin temperature and appearance of cyanosis. A few minutes before release of the rubber band, trypan blue was injected intravenously, and the dye accumulation in the areas above and below the former site of the rubber band was compared. In most cases, the other leg was available as a further control. In all cases, the area, wherein the tourniquet had been previously applied, rapidly and markedly accumulated the dye. This area of increased capillary permeability seemed to spread however, extending slightly above the area of original injury produced by the rubber band. The effect of asphyxia produced by this procedure upon dye accumulation is shown in table 5, where it will be

TABLE 5. EFFECT OF OBSTRUCTION OF BLOOD FLOW UPON CAPILLARY PERMEABILITY IN NORMAL AND ADRENALECTOMIZED RATS

Type	No. of Rats	Hours of Obstruction	Number of Rats with Increased Dye Accumulation in Asphyxiated Area
Normal rats	6	0.25	0
	6	0.5	0
	6	0.75	0
	12	1.0	0
	4	2.0	0
	4	3.0	0
	9	4.0	4
	5	5.0	3
	9	6.0	6
Bilaterally adrenalectomized rats	8	0.5	0
	12	1.0	0

seen that asphyxia of a duration of less than 4 hours is without effect. However, it appears that asphyxia of 4, 5, or 6 hours irregularly produced marked increase in capillary permeability. The objection may be raised that the legs damaged by prolonged periods of asphyxia, do not show increased dye accumulation because the blood flow to the limbs is markedly reduced. Although there may, indeed, be a reduced blood flow following damage, the finding that, after prolonged asphyxia of 4 or more hours, increased dye accumulation is obtained, demonstrates that, given an adequate stimulus, increased dye accumulation results despite decreased blood flow. The alternative to this explanation would necessitate an assumption that blood flow is increased by asphyxia after an initial decrease.

It is clear from these findings that long periods of asphyxia are without effect on capillary permeability of skin and muscle. However, if the asphyxia is of prolonged duration (4 to 6 hours) the tissue damage produced may increase capillary permeability, probably by damage and rupture of capillary walls. To check the possibility that the capillaries, in the absence of the adrenals, are hyper-sensitive to asphyxia, completely adrenelectomized rats were similarly tested. These results are also shown in table 4, and reveal that asphyxia produced by obstruction for 0.5 and 1.0 hour is without effect on capillary permeability. Since adrenalectomized rats may die in shock as soon as 0.75 hour after trauma, it is clear that anoxia acting for these time intervals would produce no increase of capillary permeability and thus fluid loss through this hypothetical mechanism appears to be of little importance in the development of this type of tramatic shock in rats.

Comment. The findings presented in this section provide strong evidence against the concept that anoxia rapidly increases capillary permeability. It appears that the duration and degree of asphyxia required to produce capillary damage in vivo, has been in general underestimated. Thus the data reported here is in agreement with the finding that traumatic shock is not accompanied by increased capillary permeability even though some degree of anoxia may be present. The effect of anoxia upon capillary permeability in man appears to warrant critical re-examination in view of our findings in rats, particularly since instances of clinical anoxia are not necessarily associated with generalized capillary damage and tissue edema.

Relation of Rate of Fluid Loss to Survival of Completely Adrenalectomized Rats

In considering the mechanism of shock due to trauma in completely adrenalectomized rats, the question arises whether there is a relationship between the rate of fluid loss and rate of onset of death. In the course of the experiments described in the

section concerned with the effect of trauma upon capillary permeability in the localized area of injury, it was discovered that the rate of edema formation in both normal and adrenalectomized rats was markedly inhibited by tramatization in the presence of an obstruction of blood flow which subsequently was removed. No explanation is advanced for this finding. However, this procedure of reducing the rate of fluid loss was utilized in studying the effect of reduction of rate of local fluid loss upon the survival time of traumatized completely adrenalectomized rats. Six adrenalectomized rats were treated in the following manner. While anesthetized with ether, rubber band tourniquets were placed around both legs, which were then traumatized in the usual way. A few minutes thereafter, the rubber bands were removed and the survival times were noted and compared to those of adrenalectomized rats, which had received identical trauma without obstruction. Four of the 6 traumatized obstructed rats died within 15 to 24 hours, while 2 survived. It will be recalled from an earlier section that all of the adrenalectomized rats damaged in the usual manner died within 6 hours. It thus appears that reduction of the rate of fluid loss is associated with an increase in survival time, and that completely adrenalectomized rats may actually survive trauma without death.

DISCUSSION

The normal rat appears highly resistant to traumatic shock produced by beating the hind legs. This resistance seems to be quantitatively related to the functional capacity of the adrenal glands, since unilaterally as well as bilaterally adrenalectomized, but not sham-operated rats, die in shock following trauma. Although death need not follow trauma in the absence of the adrenal glands, if the rate of fluid loss from the vascular system is sufficiently slow, rapid loss of vascular fluid apparently requires quantitatively adequate adrenal activity to withstand shock and death.

The manner in which the adrenal glands protect rats from the development of shock after severe trauma to the hind limbs has not as yet been elucidated. However, results to be reported subsequently indicate that the administration of desoxycorticosterone to bilaterally adrenalectomized rats raises the resistance of these treated animals. It would, therefore, appear that the adreno-cortical hormones are the effective agents of the adrenal gland for protection against this type of traumatic shock.

The initiation of shock in completely adrenalectomized rats by trauma to the hind legs is not accompanied by increased capillary permeability to trypan blue, except possibly to a slight extent in skin and to a significant degree in the area of injury. The increased capillary porosity in the damaged area is, however, of transient duration, possibly because the injured capillaries are blocked mechanically. Although it is clearly recognized that these results need not apply to man, the results of our study of capillary permeability are not necessarily at variance with the facts regarding capillary permeability in clinical shock. If shock in human beings were actually associated with a generalized increase of capillary permeability then the administration of plasma would result in passage of protein into the tissue fluids of all organs. In the absence of rapid removal of this protein from the extra-cellular fluid compartment, this tissue fluid protein would upset fluid exchange so that generalized edema would result. It is clear that plasma administered in the face of a generalized increase of capillary permeability would contribute little to the volume of the circulating blood except to cause further reductions. However, the established therapeutic value of plasma in shock is well known, and the dire consequences which presumably would follow if capillary permeability were actually increased in shock do not arise. For these reasons, it appears that clinical shock like this type of traumatic shock in rats, is not accompanied by a generalized increase of capillary permeability.

No evidence has been obtained for a vicious cycle in rats which operates to produce generalized increase in capillary permeability through the intermediation of either anoxia or of a toxic substance. On the contrary, direct evidence is presented that periods of asphyxia greater than the time intervals required to produce death are without significant effect on capillary porosity in either skin or muscle in damaged adrenalectomized rats. Investigation of the effects of generalized asphyxia, produced by allowing rats to use all of the available O_2 in a closed jar, revealed that no tissue responds to this asphyxial stimulus with increased capillary permeability to trypan blue.

It is recognized that the findings demonstrated in the rat need not necessarily apply to other types of shock in rats or other species. However, those results which are not in harmony with generally accepted ideas must be tested experimentally for their validity.

SUMMARY

Rats are normally resistant to trauma of the hind legs, which in other species produces shock and death. This high degree of resistance observed in rats is quantitatively, and not qualitatively, dependent upon adrenal function. The development of shock in rats as a result of trauma to hind limbs occurs when there is a quantitative insufficiency of adrenal function (since both unilaterally and bilaterally adrenalectomized rats are sensitive to this type of shock) and when the rate of fluid loss from the vascular system is rapid. When the loss of fluid into the area of injury is sufficiently slow, adrenalectomized rats may withstand trauma without mishap. However, if the rate of fluid loss is rapid, a quantitatively adequate degree of adrenal function is necessary to set protective compensatory reactions into motion.

Studies of the changes in capillary permeability associated with shock in injured adrenalectomized rats revealed that capillary porosity to trypan blue was normal in all tissues except in the damaged limb and possibly in skin. This increased capillary permeability in the local area of injury, however, was manifest for only a short period of time, presumably because the injured capillaries were blocked mechanically.

Re-investigation of the effects of anoxia upon capillary porosity revealed that generalized asphyxia (produced by rebreathing air) terminating in death was without effect on the permeability of the capillaries of any tissue. Studies in skin and muscle revealed that the capillaries in these tissues are able to withstand severe local anoxia for prolonged periods of time without an increase in capillary permeability. Only by local asphyxiation for 4 or more hours was it possible to demonstrate that anoxia increases capillary permeability.

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RELATION OF THYROID TO MAMMARY GLAND STRUCTURE IN THE RAT WITH SPECIAL REFERENCE TO THE MALE

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While there have appeared in the literature numerous reports on the relation of the thyroid gland to lactation, few of these have been concerned with the relationship of the mammary gland structure and the thyroid. That the thyroid is not essential for function of the mammary glands was shown by Nelson and Tobin (1), who reported that rats, thyroidectomized prior to or during pregnancy, were able to give birth to and raise normal litters. Most of the other workers, however, have found that thyroidectomy interferes with the normal mammary function (2–5). Weichert, Boyd and Cohen (6) reported marked stimulation of the mammary glands, but without lobule formation, in rats made pseudopregnant by thyroid feeding. In normal pregnant rats, Weichert and Boyd (7) showed that induced hyperthyroidism resulted in marked mammary stimulation and the appearance of secretion in the alveoli at a period earlier than it would normally occur. That mammary growth can take place in the hypothyroid state was reported by Nelson and Hickman (8), but no comparisons were made with adequate controls. They reported mammary development following estrogen treatment in either thyroidectomized or thyroidectomized castrated rats. Karnofsky (4) showed that thyroidectomy before conception or during pregnancy did not influence mammary structure.

A more extensive study was made by Leonard and Reece (9) on the influence of thyroidectomy in normal, castrated, and estrogen-treated castrated female rats. Under all conditions, thyroidectomy resulted in stimulation of the alveolar system and thickening of the ducts. They reported an increase in the number of end-buds after thyroidectomy, but their plates do not convincingly indicate this point. Injection of thyroxin tended to prevent the changes resulting from thyroid removal, which showed that the thyroid hormone was probably the active principle concerned. Unfortunately, in none of these studies was a clear distinction made between the types of mammary growth obtained.

The results of Leonard and Reece suggested a similar study in the young male rat in order to make a more careful analysis and to determine whether the male mammary gland behaved in a manner similar to that of the female. Further, it seemed advisable to study more extensively the type of mammary structure resulting from interference with the normal function of the thyroid. In the following experiments an attempt was made *a*), to confirm the work of Leonard and Reece on the effect of thyroidectomy in immature female rats, *b*) to compare the mammary growth of normal and castrated male rats with and without thyroids, and *c*), to compare mammary growth with and

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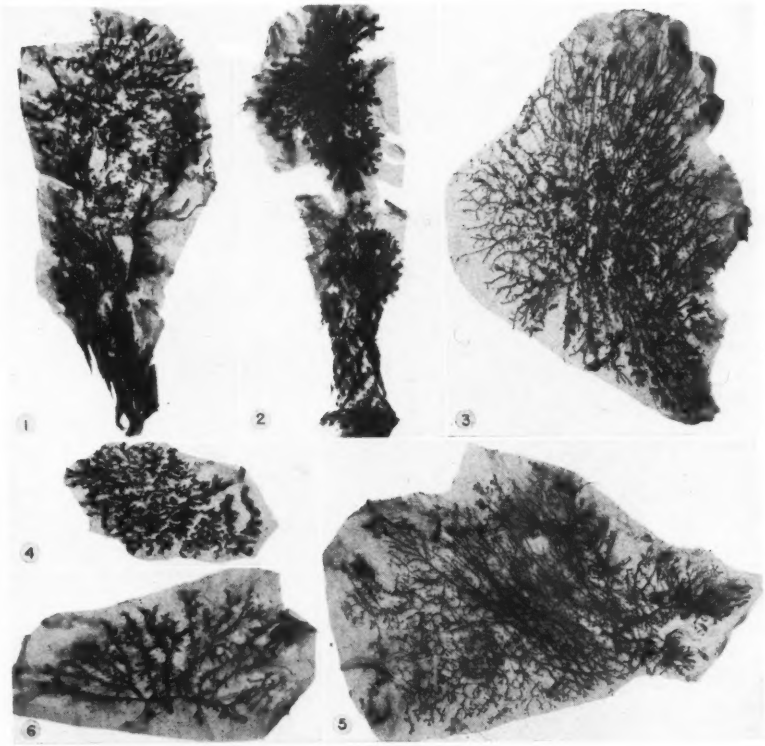

Fig. 1–6. WHOLE MOUNTS OF MAMMARY GLANDS FROM RATS. Fig. 1. Immature female rat injected with menopause urine extract. Fig. 2. Thyroidectomized immature female injected with menopause urine extract. Fig. 3. Normal male rat. Fig. 4. Thyroidectomized male. Fig. 5. Castrated male. Fig. 6. Thyroidectomized castrated male.

without treatment with certain sex hormones. The results of study on these problems are presented here.

METHODS

Normal and castrated albino male rats of the Sprague-Dawley strain were thyroidectomized at 25 days of age. They were fed a ration recommended by Maynard (10).[2] Food was given *ad libitum* and supplemented with 0.5 per cent calcium lactate to prevent tetany which sometimes occurs in the rat when the parathyroids are removed together with the thyroids. Alpha-estradiol dipropionate and testosterone propionate[3] were used to stimulate mammary growth in the castrated rats. Body weighings were made before and at the termination of the experiments. The right abdominal mammary gland from each animal was stained and mounted *in toto* for comparison. Examination for remnants of thyroid tissue was made under the binocular microscope at autopsy.

[2] Calf meal ration purchased from G. L. F. Cooperative, Ithaca, N. Y.

[3] Dr. R. MacBrayer, Ciba Pharmaceutical Products, Inc., Summit, N. J., generously supplied the hormones, α-estradiol dipropionate (Di-ovocylin) and testosterone propionate (Perandren).

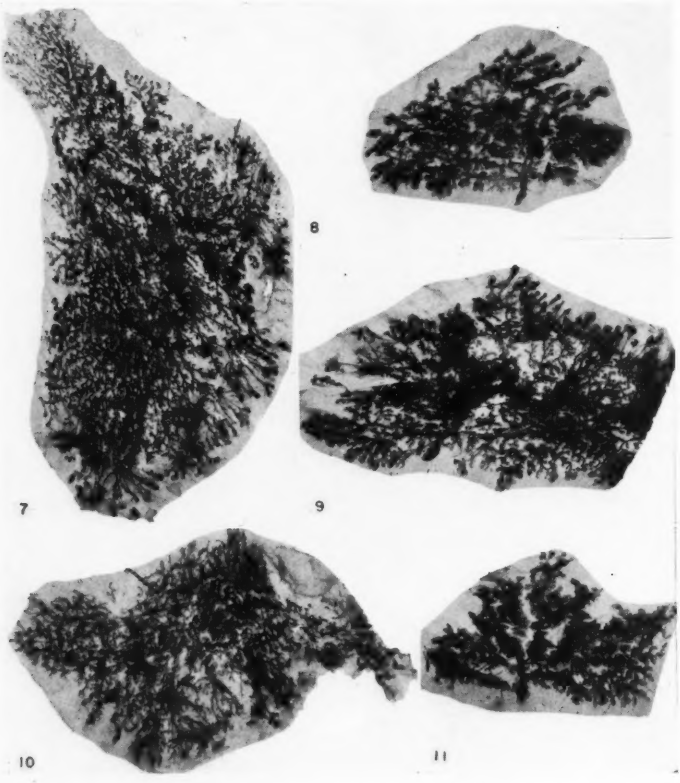

Fig. 7–11. WHOLE MOUNTS OF MAMMARY GLANDS FROM RATS. Fig. 7. Castrated male injected with estrogen. Fig. 8. Thyroidectomized castrated male injected with estrogen. Fig. 9. Incompletely thyroidectomized castrated male injected with estrogen. Fig. 10. Castrated male injected with male hormone. Fig. 11. Thyroidectomized castrated male treated with male hormone.

RESULTS

In order to confirm the results of Leonard and Reece on the effect of thyroidectomy in the female, 8 of 16 immature female rats were thyroidectomized. After a lapse of 4 days, all were injected with an extract of a tannic acid precipitate of menopause urine in amounts, which over a period of 4 days, more than trebled the size of the ovaries. Comparison of the mammary glands revealed that, after thyroidectomy, a marked thickening of the ducts occurred, but the total extension of the mammary tree was reduced (fig. 1, 2). The most marked result was a great increase in lobular-alveolar proliferation after thyroidectomy, similar to that obtained by the above mentioned workers.

Effect of thyroidectomy on mammary structure of normal male rats. Four 25-day old males were thyroidectomized and 4 were kept as controls. After a period of 35 days, the rats were killed and the right abdominal mammary gland removed. Observations on the body weight showed that the thyroidectomized animals gained an average of 70 gm. during the experimental period in comparison with 177 gm. for the controls.

The mammary glands of all the control animals showed a normal extension of the duct system and normal lobular-alveolar proliferation (fig. 3). All of the completely thyroidectomized animals showed only a poor to fair extension of the duct system with apparently no active end-bud growth. Thickening of the ducts was evident, and a definite lobular-alveolar system was observed which was superior to that of the controls (fig. 4). The gland from one incompletely thyroidectomized rat showed an intermediate condition, with greater extension of the duct system than in the cases of complete thyroidectomy, but with less alveolar development.

Effect of thyroidectomy on mammary structure of castrated male rats. Twelve 24-day old males were castrated, and 6 of these were thyroidectomized the day following. After a period of 41 days, the control rats were found to have gained an average of 140 gm. as compared with 67 gm. for the thyroidectomized group.

Examination of the mammary glands showed distinct differences similar to those observed in the previous experiment. All of the control glands showed good extension of the duct system, but no evidence of alveolar development (fig. 5). All of the glands from the thyroidectomized animals showed very poor extension of the duct system, but a definite thickening of the ducts and what might be considered a slight alveolar development was observed (fig. 6).

Effect of thyroidectomy on mammary structure of estrogen treated castrated male rats. As above, 12 males were castrated. Six of these were thyroidectomized and 6 retained as controls. Of the thyroidectomized group, one died and one was subsequently determined to be incompletely thyroidectomized. After a period of 30 days, all were injected with 5 micrograms of α-estradiol dipropionate in 0.05 cc. of olive oil daily for 10 days. At autopsy the day following the last injection, the controls were found to have gained an average of 113 gm. compared with 37 gm. for the experimental group (excluding the one animal incompletely thyroidectomized.)

The mammary glands were even more easily separated into two distinct groups than in the case of the non-injected animals. All of the control glands showed typical estrogen stimulation, with excellent extension of the duct system and end-bud development, but with little or no alveolar growth (fig. 7). The four completely thyroidectomized rats had glands showing very poor duct extension with apparently no end-bud growth, but very good lobular-alveolar development. The ducts were observed to be very much thickened, and lobulation was dense with extreme dilatation of the alveoli (fig. 8). The one incompletely operated rat had a mammary gland of an intermediate type. The duct system was much more extensive than in the completely operated individuals, and the ducts were less thickened. Good end-bud growth was evident, but the lobular-alveolar system was less well developed (fig. 9).

Effect of thyroidectomy on mammary structure of male hormone treated castrate male rats. Five thyroidectomized and 5 control castrated male rats were used. After a latent period of 30 days, all were injected with 250 micrograms of testosterone propionate in 0.05 cc. of olive oil daily for 10 days. At autopsy the day following the last injection, the controls were found to have gained 156 gm. as compared with 49 gm. for the experimental group.

Decided differences were found between the mammary glands of the two groups All of the controls showed a fair extension of the duct system and a fair development of the alveolar system, with a degree of lobulation typical of testosterone stimulation (fig. 10). All of the glands of the thyroidectomized rats showed poor extension of the ducts with no end-bud growth. Considerably more lobular-alveolar development was found, however, in which all showed dense lobulation with thickened ducts, presenting an exaggerated effect of male hormone (fig. 11).

The most striking result was noted in the great similarity between the mammary glands of the thyroidectomized rats treated with estrogen and with male hormone. It was impossible to tell the glands apart; the glands of the estrogen-treated rats appeared as if they came from rats treated with male hormone.

DISCUSSION

The preliminary experiment to confirm the work of Leonard and Reece (9) on the effect of thyroidectomy on the female mammary gland, as well as the present work on the male, showed the general results of thyroidectomy not only to be an inhibition of duct extension, but also a stimulation of lobular-alveolar development and a thickening of the ducts. The present findings are essentially in agreement with the results of these authors, in that the principal effect reported by them was a considerable increase in alveolar development after thyroidectomy. However, they failed to make a sharp distinction between duct and alveolar development. That such a distinction is necessary is indicated by the present work. It should be pointed out that they used sexually mature females with the much more extensive duct system characteristic of the female, which probably confused their observations. In the present study, the glands of the immature females and males were obviously capable of greater growth during the experimental period, and any inhibitory influence upon growth would be more easily detected.

In the normal and castrated males it was noted that the duct end-buds were bulb-like, indicating continued duct extension. In the males deprived of their gonads this may have been the result of the action of the pituitary mammogen (12, 13) or some extra-gonadal source of estrogen, *i.e.* adrenal cortex. After thyroidectomy in both normal and castrated animals, the bulb-like end buds were not observed.

In the castrated males treated with male hormone, the end-bud growth may have been present, but the thickening of the ducts obliterated the contrasting picture seen in the previous case. It was noted that the total areas of the mammary glands from the castrated rats treated with male hormone were not as great as those of the castrated controls. Comparison of figures 5 and 10 bear this out. It is possible that the male hormone actually inhibited further duct extension during the 10-day period of treatment. Observations on the young normal male approaching sexual maturity indicate a cessation of duct extension (11). After thyroidectomy, however, a very radical inhibition of duct extension appeared in all cases. In many glands secretory material was observed within the enlarged alveoli.

It is to be emphasized that in studies of this kind, a careful analysis of the types of mammary growth obtained should be clearly stated. Male hormone normally stimulates the lobular-alveolar system, and in all of the experiments using testosterone propionate, this was found to be the case. After thyroidectomy, this effect was markedly accentuated. Furthermore, this increased lobular-alveolar growth occurred simultaneously with an inhibition of the duct extension in the operated animals. However, the appearance of duct thickening, increased lateral duct-buds and the tendency toward alveolar formation in the thyroidectomized castrated rats is difficult to explain. If one can accept the pituitary mammogen as a general mammary growth stimulator, it is possible that the absence of the thyroid can modify its influence as well as that of the sex hormones. Further work on this point is indicated.

In contrast to the action of male hormone in normal and thyroidectomized rats, estrogen produced an entirely different effect from that which one would expect after thyroid removal. If the characteristic effect of estrogen in castrated rats is to extend the duct system, it would seem logical to expect that an accentuated duct

extension might result. Instead, however, thyroidectomy induced lobular-alveolar development to a degree indistinguishable from that produced by the male hormone at the doses given. The differential action of the two hormones seen in normal castrates, disappeared after thyroidectomy; α-estradiol dipropionate, under these conditions, had the same effect as testosterone propionate. Some secretory material was present also in the large alveoli of these glands.

The intermediate condition of the glands in cases of incomplete thyroidectomy is a particularly clear indication of the effect of the thyroid and its removal upon mammary growth inasmuch as both the effect of its partial presence and its absence can be seen in these glands (fig. 9).

The action of the several hormones in relation to thyroidectomy is a problem that at present is unexplained and must be analyzed further. Since it is known that male hormone or gonadotropic substances will produce lobular-alveolar development (11), it is not surprising that considerable alveolar development should be obtained in the present study with such treatment. Thyroidectomy, it might be argued, serves merely to increase the effectiveness of these hormones, whether they be of endogenous or exogenous origin. With estrogen, however, this cannot be the entire explanation, for although various degrees of alveolar development may be obtained in the normal castrate rat with large doses of the hormone, complete lobulation does not occur (14). It is not likely that the lobular-alveolar proliferation obtained after thryoidectomy with subsequent estrogenic treatment can be attributed solely to an increased effectiveness of the estrogen. This is all the more evident in the light of the failure of the estrogen to cause duct growth, its most characteristic effect.

After thyroidectomy, estrogen and testosterone appear to be equipotential in their effect on mammary growth. Astwood, Geschickter and Rausch (11) report that overdosage with either estrogen or testosterone will produce an abnormal type of gland with thickened ducts, enlarged lateral end buds and cyst formation. Doses greater than 50 μg. of α-estradiol dipropionate or of 1.33 mg. of testosterone propionate daily for 20 to 60 days were used to produce this abnormal development. These levels are so much in excess of those used in the present work (5 μg. of estradiol dipropionate or 0.25 mg. of testosterone propionate daily for 10 days) that no comparison of results is possible. Furthermore, instead of presenting an abnormal appearance, some glands obtained in the present work appear to be in a normal secretory state. That we are not dealing with the effect of a given amount of hormone on a smaller mammary gland was shown by another experiment. A normal response to estrogen was obtained in a mammary gland of a reference control, which was the same size as the operated animals given the same hormone dosage.

Another explanation is necessary for this apparent reversal of the properties of estrogen, and at present, it is only a conjecture. An antagonistic relationship is known to exist between thyroxin and estrogen. Hyperthyroidism increases the threshold of response to estrogen (15) and injection of estrogens will lower the basal metabolic rate of rats as much as 28 to 54 per cent, while after marked elevation of the metabolic rate by thyroid extract, the return to normal can be hastened by the injection of estrin (16). The difference in the effect of estrogen after thyroidectomy may be more than quantitative. Thus, it is possible that estrogen may have the potential power to produce both duct and alveolar growth, but the power to produce the latter may be held in check by the normal function of the thyroid. This may explain why removal of the thyroid promotes alveolar development, and in addition, apparently inhibits duct extension. In the experiments using testosterone propionate, or in the normal males, the situation appears that thyroidectomy augments the normal alveolar-growth-

promoting effects of the male hormone, and inhibits the duct growth potentialities inherent within the animal. In the immature animal with an intact thyroid, it appears that duct extension is the most characteristic aspect of mammary growth, while removal of the thyroid at this stage inhibits duct extension and hastens alveolar development.

SUMMARY

Thyroidectomy in normal immature male rats resulted in inhibition of mammary duct growth, and stimulation of alveolar development, when compared with their controls.

Thyroidectomy in castrated males brought about much the same structural changes, but to a lesser degree, when comparison was made with castrated controls.

Thyroidectomized, castrated males treated with testosterone propionate had mammary glands with an exaggerated lobular-alveolar development; there was an inhibition of duct growth, when comparison was made with appropriate controls.

Thyroidectomized castrated males treated with α-estradiol dipropionate gave results indistinguishable from those obtained in the group treated with testosterone propionate. Thus, under these conditions, α-estradiol dipropionate and testosterone propionate exhibit similar effects on mammary growth.

The general effects of thryoidectomy on mammary growth in immature rats are *a*), an inhibition of duct extension, *b*), the production of a small compact type of gland with thickened ducts and *c*), a marked development of the lobular-alveolar system.

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RELATION OF MAMMOGENIC LOBULE-ALVEOLAR GROWTH FACTOR OF THE ANTERIOR PITUITARY TO OTHER ANTERIOR PITUITARY HORMONES

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IT IS NOW generally acknowledged that the anterior pituitary as well as the sex hormones plays a vital rôle in the processes of mammary gland growth. Estrogen, especially, has been studied as to its rôle with pituitary materials in stimulating mammary gland growth. Reece and Leonard (1) have suggested that, in hypophysectomized rats, either the pituitary growth extract sensitized the mammary gland to the action of estrogen to secure growth, or the estrogen sensitized the mammary gland to the action of the pituitary extract. Gardner and White (2) in similar experiments with hypophysectomized male mice suggest that the lactogenic extract they used sensitized the mammary gland to the action of estrogen. Mixner and Turner (3) in an extensive series of experiments have investigated the rôle of estrogen in the stimulation of mammary lobule-alveolar growth by progesterone and the mammogenic lobule-alveolar growth factor of the anterior pituitary. They conclude that estrogen acts directly on the mammary gland, increasing the permeability of the capillaries and tissues and producing a hyperemia and increased vascularity such that the pituitary mammary-growth stimulus can be most effective.

Of considerable importance, also, in this study of pituitary factors which affect mammary gland growth, is the exact nature of this factor or factors. Are there specific pituitary mammogenic factors which are different from the identified or known pituitary factors? Are these mammary growth effects due to several known pituitary factors or to a single known factor?

Gomez and Turner (4, 5), both on the basis of the normal physiology of mammary gland growth and on the basis of hypophysectomy and replacement therapy experiments, have postulated the presence of two pituitary mammogens, a mammogenic duct-growth factor, stimulated to secretion by estrogen and a mammogenic lobule-alveolar growth factor, stimulated to secretion by progesterone and estrone.

A priori considerations would indicate that the lactogenic hormone is not responsible for mammary lobule-avleolar growth, as many studies (6, 7) have indicated that the pituitary secretion of the lactogenic hormone during pregnancy does not appreciably rise above the level of that of the growing animal until parturition takes place, after which, or at this time, it rises very sharply. Thus, mammary lobule-alveolar growth occurs at a time during pregnancy when there is no significant rise in the level of the lactogenic hormone secretion of the anterior pituitary.

The development of sensitive technics for assaying pituitary materials and ex-

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tracts for their potency in mammogenic, lactogenic, thyrotropic and gonadotropic hormones has made possible a quantitative study of the relation of the mammogenic lobule-alveolar growth factor to several of these other anterior pituitary hormones.

The purpose of this paper, then, is to compare the mammogenic lobule-alveolar growth potencies of a group of pituitary extracts with the lactogenic, thyrotropic and gonadotropic hormone potencies. These extracts vary rather widely in the potency of the principal hormone. Such a comparison should reveal whether or not the mammogenic fraction is identical with, or is primarily associated with, any one of these other known pituitary hormones.

PROCEDURE

A group of anterior pituitary materials and extracts were assayed for their properties in promoting mammary lobule-alveolar growth (3, 8), using the ovariectomized female mouse as the assay animal. Estrone (75 I.U. per mouse per 10 days) was given in addition to the extracts to be assayed. The extracts varied from fresh cattle anterior pituitary to a very highly purified lactogenic extract[3] (30 I.U. per mg.) and also to an extract very high in thyrotropic and gonadotropic hormone potency. Most of these extracts were assayed for the potency of the lactogenic hormone (I.U. per mg.) by the method of McShan and Turner (9, 10). A few were assayed for the thyrotropic and gonadotropic hormone content using the Bergman-Turner chick assays (11, 12). Lactogenic, thyrotropic and gonadotropic hormone assays were made by A. J. Bergman who also prepared most of the extracts used in this study.

EXPERIMENTAL RESULTS

In some earlier experiments (13) mammary lobule-alveolar growth was secured with fresh anterior pituitary tissue from pregnant cattle while such growth was not secured with a lipid extract of this material. In subsequent reports (3, 14) lobule-alveolar growth has been secured with both actone-ether dried pituitary and with an initial extract prepared according to a method described by Bergman and Turner (15). These extracts are definitely protein in nature which thus associates this factor with the other protein-like hormones of the anterior pituitary.

Lots 14 and 15 of pregnant, and one lot, 41, of mixed, cattle pituitaries were assayed in both the fresh and the acetone-ether dried condition (table 1) for their potency in promoting mammary lobule-alveolar growth. One part of acetone-ether dried material was secured from approximately 5 parts of fresh tissue. *Lot 41* was further extracted to an initial extract, with modifications, according to the method of Bergman and Turner (15). One part of initial extract was secured from approximately 14 parts of acetone-ether dried material or from 70 parts of fresh tissue. This was also assayed for activity in producing mammary growth.

An examination of the assay levels of response in table 1 shows that upon drying the fresh pituitary with acetone-ether, approximately 50 per cent of the potency of fresh pituitary is present in the acetone-ether dried protein material. On further extraction to the initial extract (*lot 41*), approximately 8 per cent of the potency in the original fresh tissue is recovered in this initial extract. The question may arise here as to whether these are 'true potencies' or only 'apparent potencies.' Differences in solubility and rates of absorption of the various materials enter into this question.

In the second group of experiments (table 2) a wide variety of extracts were again assayed for their properties in promoting mammogenic lobule-alveolar growth. Some

[3] Two lactogenic hormone extracts were supplied by the Schering Corp., Bloomfield, N. J., through the courtesy of Dr. E. Schwenk.

of these extracts were very high in their content of lactogenic hormone, while one, *thyr. 2a-40*, was especially high in the thyrotropic and gonadotropic hormones. Table 3, which includes data on extracts assayed as shown in tables 1 and 2, shows the results of comparative assays of mammogenic and lactogenic hormone content in

TABLE 1. LOBULE-ALVEOLAR RESPONSES OF CASTRATE FEMALE MICE TO VARIOUS CATTLE ANTERIOR PITUITARY MATERIALS INJECTED FOR 10 DAYS

No. of Mice	Pituitary Material Total Dosage, Mg.	Lobule-Alveolar Responses +	Lobule-Alveolar Responses −	Per Cent Positive	Mouse Units per 3.5 gm. of Fresh Tissue, Equivalent
		Lot 14, pregnant cattle, fresh pituitary			
12	150	12	0	100.0	
14	100	14	0	100.0	
15	50	13	2	86.7	
13	35	7	6	53.8	100
		Lot 15, pregnant cattle, fresh pituitary			
13	35	6	7	46.1	100
		Lot 41, mixed cattle, fresh pituitary			
13	100	13	0	100.0	
13	50	8	5	61.5	80[1]
		Lot 14, acetone-ether dried pituitary			
12	30	11	1	91.2	
13	15	6	7	46.1	46
		Lot 15, acetone-ether dried pituitary			
14	15	8	6	57.1	46
		Lot 41, acetone-ether dried pituitary			
13	20	8	5	61.5	40[1]
		Lot 41, initial extract			
34	17.5	33	1	97.0	
23	12.5	20	3	87.0	
21	10.0	12	9	57.1	
35	7.5	18	17	51.1	6.7

[1] Corrected to basis of 50 per cent positive responses from 61.5 per cent.

these extracts. As may be seen from the right column in table 3 the I.U. of lactogen per mammogenic unit ranges widely from approximately 2 to 352, a differential of 176. Thus these extracts vary widely in their relative content of mammogenic and lactogenic materials. The more potent lactogenic extracts required extreme amounts of extract, on a lactogen basis, as compared to the less potent extracts, to secure mammary lobule-alveolar growth. Of course it is possible that, in the more crude extracts of the pituitary, such synergistic relations may exist between the active mammary growth factor and other pituitary hormones that much less of the active principle is

required. However, it is not thought that this synergism could be so great as to explain the mammary growth obtained in these experiments on the basis of lactogen as the active mammogenic factor. It is more probable that the mammogenic and lactogenic factors are separate entities and that even in the purest amorphous lactogenic extract assayed,[3] impurities of a mammogenic factor are present.

TABLE 2. LOBULE-ALVEOLAR RESPONSES OF CASTRATE FEMALE MICE TO VARIOUS ANTERIOR PITUITARY EXTRACTS INJECTED FOR 10 DAYS

No. of Mice	Pituitary Material		Lobule-Alveolar Response		Per Cent Positive
	Extract No.	Total dosage in mg.	+	−	
4	Cattle *Lac.* #39	40.0	4	0	100.0
4	Cattle *Lac.* #39	20.0	4	0	100.0
22	Cattle *Lac.* #39	12.5	20	2	90.9
12	Cattle *Lac.* #39	10.0	5	7	41.7[1]
3	Cattle *Lac.* #39	5.0	0	3	0.0
10	Schering 46-51$_{52}$ 4	15.0	8	2	80.0
10	Schering 46-51$_{52}$ 4	10.0	5	5	50.0[1]
5	Schering 48 *hy-ex-4*	25.0	5	0	100.0
5	Schering 48 *hy-ex-4*	15.0	5	0	100.0
11	Schering 48 *hy-ex-4*	7.5	6	5	54.5[1]
3	*L-1a-40*	50.0	3	0	100.0
4	*L-1a-40*	37.5	3	1	75.0
4	*L-1a-40*	25.0	3	1	75.0
8	*L-1a-40*	20.0	4	4	50.0[1]
5	*L-1a-40*	12.5	1	4	20.0
11	*L-1a-40*	10.0	0	11	0.0
11	*Cl$_3$-41-70*	13.5	5	6	45.5[1]
12	*Cl$_3$-41-70*	10.0	4	8	33.3
12	*L-1-41-60*	15.0	6	6	50.0[1]
13	*L-1-41-70*	20.0	8	5	61.5[1]
13	*Thyr. 2a-40*	10.0	0	13	0.0

[1] Assay level of responses.

The case is more complete for the separateness of the mammogenic factor from the thyrotropic and gonadotropic hormones. A thyrotropic-gonadotropic extract, *thyr. 2a-40* (table 2), which contained 10 chick units of thyrotropic hormone per mg. and 4 chick units of gonadotropic hormone per mg., failed to elicit any mammary growth when assayed at 10 mg., a total of 100 C.U. of thyrotropic and 40 C.U. of gonadotropic hormone. These amounts of hormones greatly exceed that amount present in a mammogenic unit of any of the other extracts assayed. Thyrotropic and gonadotropic assays were available for three of the extracts assayed for mammogen (tables 1 and 2). The results of comparative assays of the mammogenic, thyrotropic and gonadotropic content of these extracts are presented in table 4. The chick units of thyrotropic hormone per mammogenic unit varied from 1.31 to 22.07, a differential of 16.9 while the chick units of gonadotropic hormone varied from .095 to 5.72, a differential of 60. It is thus rather apparent that the mammogenic factor is not associated with either the thyrotropic or gonadotropic hormones.

Extracts were not available which were known to consist predominantly of

TABLE 3. COMPARATIVE MAMMOGENIC AND LACTOGENIC HORMONE ASSAYS ON A GROUP OF ANTERIOR PITUITARY EXTRACTS

Extract	Mg. Extract per Mammogenic Unit	I.U. of Lactogen per Mg. of Extract	I.U. of Lactogen per Mammogenic Unit
Lot 14, fresh cattle pituitary	35	.06	2.1
Lot 15, fresh cattle pituitary	35	.06	2.1
Lot 41, fresh cattle pituitary	44[1]	.06	2.6
Lot 14, acetone ether dried	15	.3	4.5
Lot 15, acetone ether dried	15	.3	4.5
Lot 41, acetone ether dried	17.6[1]	.3	5.1
Lot 41, initial extract	7.5	4.5	33.8
Cattle Lac. #39	10.0	4.0	40.0
Schering 46-51-p₂ 4	10.0	12.0	120.0
Cl₂-41-70	13.5	12.0	162.0
Schering 48-hy-ex-4	7.5	30.0	225.0
L-1a-40	20.0	12.0	240.0
L-1-41-60	15.0	18.0	270.0
L-1-41-70	17.6[1]	20.0	352.0

[1] Corrected to basis of 50 per cent positive responses from 61.5 per cent.

adrenotropin or growth hormone so it was not possible to make any comparison of the mammogenic factor to these two factors.

DISCUSSION

As the extraction and purification methods for the various pituitary hormones become more efficient in the direction of purity and non-contamination by other hormones, the tendency is going to become more common to ascribe all physiological effects of these preparations to the principal hormone involved. Gardner and White (2) have secured mammary duct growth in hypophysectomized mice with a highly purified lactogenic hormone preparation plus estrogen. Similarly Lyons, Simpson and Evans (16) have secured mammary lobule-alveolar growth in hypophysectomized rats with a lactogenic hormone preparation plus estrogen. That the active mammogenic principle involved here is the lactogenic hormone does not follow.

A highly purified lactogenic preparation[3] (30 I.U. per mg.) had considerable potency for promoting mammary lobule-alveolar growth in ovariectomized female mice, 7.5 mg. being required for our assay response, a total of 225 I.U. of lactogen. In other less pure preparations of lactogen, the number of I.U. of lactogen per mammogenic unit varied from about 350 down to 2 I.U. for the fresh pituitary itself,

TABLE 4. COMPARATIVE MAMMOGENIC, THYROTROPIC AND GONADOTROPIC HORMONE ASSAYS ON A GROUP OF ANTERIOR PITUITARY EXTRACTS

Extract	Mg. Extract per Mammogenic Unit	Thyrotropic[2]		Gonadotropic[2]	
		C.U. per mg. extract	C.U. per mammogenic unit	C.U. per mg. extract	C.U. per mammogenic unit
Cl₁-41-70	13.5	0.097	1.31	0.007	0.095
Lot 41, acetone ether dried	17.6[1]	0.278	4.89	0.110	1.936
Lot 41, initial extract	7.5	2.943	22.07	0.776	5.72

[1] Corrected to basis of 50 per cent positive responses from 61.5 per cent.
[2] Chick units.

indicating quite definitely that lactogen is not the active principle involved in mammary lobule-alveolar growth.

These experiments do not rule out the distinct possibility, or even probability, that other pituitary factors might synergize with the active principle to some extent in causing mammary lobule-alveolar growth. It is extremely unlikely, however, that there is anything like a 176-fold synergism of other factors with lactogen to cause the growth observed in these experiments.

These experiments indicate, then, that there is a pituitary mammogenic lobule-alveolar growth factor which is distinct from the lactogenic, thyrotropic and gonadotropic hormones. Evidence is not at hand at present to show its possible identity with the growth, adrenotropic or mammary duct growth hormones.

SUMMARY

Comparative assays for the mammogenic lobule-alveolar growth factor, lactogenic, thyrotropic and gonadotropic hormones were made on a group of anterior pituitary materials and extracts which varied widely as to the kind and amounts of hormones present in them.

The mammogenic lobule-alveolar growth factor was found to be definitely protein in nature as are the other anterior pituitary hormones.

Comparative assays indicated that the mammogenic lobule-alveolar growth factor is not identical with either the lactogenic, thyrotropic or gonadotropic hormones.

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ACTION OF DIETHYLSTILBESTROL ON CYTOLOGICAL CHARACTERISTICS OF ANTERIOR PITUITARIES OF FEMALE RATS, TOGETHER WITH CERTAIN OBSERVATIONS ON THE EFFECT OF CASTRATION[1]

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SINCE diethylstilbestrol was isolated by Dodds and his coworkers (1) this synthetic estrogen has been the subject of extensive laboratory and clinical study. Several reports concerned with its action on the structure of the anterior pituitary gland of rats recently appeared (2, 3, 4) which have indicated that the action of this substance is similar to that of the naturally occurring estrogens. Castration changes were prevented, and there was a decrease in the level of basophiles and eosinophiles, and an increase in the levels of chromophobes. The present communication is concerned chiefly with the action of this substance on the cytology of the anterior lobe cells of normal and castrated female rats, less attention being given to its effects on the proportionate numbers of the various cell types. In addition, certain structural characteristics of the anterior pituitary gland of uninjected castrated rats are analyzed, and their significance in relation to the release of gonadotropic substances from the anterior lobes of castrated rodents and human beings is considered.

MATERIALS AND METHODS

Nineteen normal female rats, approximately 25 days of age, received subcutaneously variable amounts of diethylstilbestrol in oil, ranging from 50 micrograms to 100 mg., over periods varying from 3 to 15 days. Twelve normal litter-mate controls were used. A second group of 10 rats of the same age were first castrated and given similar amounts of the synthetic estrogen for 15 days. Eleven uninjected litter mates, castrated at the same time, were used as controls. In addition, 8 female rats castrated 3 months previously were used.

At autopsy, the pituitaries were divided into halves sagittally and fixed in Champy's fluid for 24 hours. Usually one-half of each gland, following fixation, was chromated by the modified Champy-Kull method of Severinghaus (5) and used for general cytologic study. The second half, following fixation, was generally osmicated (Nassanov-Katachev method) for 5 days in an oven at 37° C., and used for the study of the Golgi apparatus. Most of the material was double-embedded by Severinghaus' procedure in paraffin (62°F. although good results have been obtained in some instances by direct embedding in 60 to 62° Tissumat (Fisher). Both the osmicated and chromated tissues were cut serially at 3 micra and stained by the technic of Fain and Wolfe (6) or modifications of this procedure.[2] By this method the mitochondria were stained brilliant fuchsin, the eosinophilic granules orange or orange-red, and the basophilic granules blue. The eosinophilic granules were prominent and unless they were very closely packed in the cell, the individual granules were easily seen (fig. 14). In partially degranulated cells the persisting granules were usually found in small

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[2] These modifications will be described later by W. R. Fain.

clumps (fig. 15). The basophilic granules in some cells were quite fine while in others they were coarsely flocculent; in either instance they were evenly distributed in the cell, regardless of their abundance. The cytoplasm of the granular basophiles usually appeared as a granular mass, but separate granules were not easily discerned (fig. 17, 18, 19). The cells in which the granules were abundant and closely packed stained a deep blue color (fig. 1 and 17); those in which they were more widely spaced were less intensely blue (fig. 19) and the non-granular basophiles were light blue (fig. 2 and 20). Throughout this study we have used this staining reaction as an aid in judging the granular content of the basophiles.

Anterior pituitaries of normal immature control rats. The abundant eosinophiles were mostly small or moderate in size and the orange or orange-red granules were generally closely packed (fig. 13). In a majority of these cells, the Golgi apparatus appeared as a network of osmiophilic strands capping the nucleus; this is the form described by Severinghaus (7) as being characteristic of eosinophiles in the rat. However, in immature rats, as has recently been pointed out by Ayers (8), there are frequent variations from this cap-like form. In many cells the Golgi apparatus formed an irregular vesicle which was usually in contact with and sometimes pressed against the nucleus (fig. 13), but in a few cells the apparatus was located some distance from the nucleus. The latter condition was usually found in somewhat elongated cells in which the nucleus was eccentrically placed; this position resulted in an increase in the amount of cytoplasm on one side of the nucleus so that the Golgi body was not necessarily pressed against the nucleus. Quite frequently one or more small clear vesicles were found in the meshes of the Golgi material (fig. 13, 14, 15). The granular mitochondria were scattered among the eosinophilic granules (fig. 13). In many cells they tended to be more abundant in the region occupied by the Golgi apparatus.

Basophiles showing all degrees of granulation were present. Those which were filled with granules were stained a deep blue (fig. 1) while the degranulated cells were light blue (fig. 2). Partially degranulated cells (fig. 4) formed intermediate variants between the dark and the light cells. The Golgi apparatus of the basophiles was usually in the form of an irregular hollow sphere (fig. 1–4), often with osmiophilic strands projecting from the wall into the center of the sphere (fig. 1). Both the wall and these strands were irregular in thickness. Usually, the Golgi body was found free in the cytoplasm some distance from the nucleus, a condition previously reported as characteristic of these cells by Severinghaus (7); however, this was not an invariable finding (see below). In many cells the small clear vesicles mentioned previously were found embedded in the meshes of the Golgi apparatus, confirming findings of previous workers (8, 9, 10).

Generally, but not invariably, the Golgi apparatus was larger and more complicated in the granular basophiles than in the non-granular (fig. 1–4). Quite often in the former the apparatus lost its obviously spherical form and was made up of a mass of anastomosing strands of variable thickness. In occasional cells the Golgi body was found to lie in close relation to the nucleus and even to form a semilunar mass about it, this condition being more common in cells in which there seemed to be insufficient room for the apparatus to lie free in the cytoplasm. In fact, in both the basophiles and eosinophiles, the form of the Golgi material seemed to be influenced, at least to some extent, by the shape of the cells and space available for it to occupy. As a whole, although variations were encountered, the Golgi apparatus appeared to be smaller and to resemble a simple sphere in the non-granular basophiles (fig. 2, 3). Many of the non-granular basophiles presented certain regressive changes, *i.e.*, the nucleus was unusually basophilic and the nuclear membrane wrinkled or crenated. Often the

cytoplasm was pulled away from the nucleus. In such cells the Golgi apparatus was generally vesicular and somewhat shrunken (fig. 3).

Mitochondria were usually abundant in all of the basophiles but they were generally more so in the partially degranulated and degranulated basophiles than in the fully granulated cells (fig. 1, 2, 4). However, variations were noted, since in some cells packed with granules mitochondria were quite abundant. In the regressive non-granular basophiles they were usually reduced in number.

Anterior pituitaries of castrated immature control rats. These animals had been castrated 15 days before death and presented castration changes in the anterior lobe which were characteristic of immature rats castrated for this period (11).

The eosinophiles in the pituitaries of this group were similar to those found in normal immature rats and need not be discribed.

There was a marked increase in the size and number of basophiles.[3] The cytoplasm of many cells contained vairable numbers of small vacuoles, although there were no signet-ring castration cells. The non-vacuolated cells were much more abundant and varied in appearance. Some were filled with granules and stained deeply (fig. 17). The Golgi apparatus in these cells was generally large; but the mitochondria tended to be less numerous than in cells containing fewer granules. The type of granular basophile most frequently found tended to be slightly larger than that just described. These cells stained less intensely and the granules, while still abundant, were less closely packed (fig. 18). The Golgi apparatus was essentially similar to that of the cells containing more granules. Mitochondria were generally, but not invariably, more abundant (fig. 18). At present it is difficult to say whether these cells were or were not beginning to lose their granules. However, numerous partially degranulated basophiles were seen (fig. 19), almost invariably characterized by abundant mitochondria.

Considerable numbers of degranulated basophiles both large (fig. 20) and small were observed. Mitochondria were generally quite abundant, particularly in the larger cells (fig. 20). Such cells always contained more mitochondria than did the deep blue basophiles packed with granules (fig. 17); however, in many instances there were no more mitochondria in these than in the less deeply stained basophiles illustrated in figure 18. In addition to the increase in number, the mitochondria in the degranulated cells often appeared slightly enlarged (fig. 20). The Golgi apparatus in these cells varied in size and form (fig. 20); generally it was smaller than in the granular cells, but sometimes it was as large. In addition to the apparently normal degranulated basophiles, a small number of regressive cells were found. These were characterized by a tendency toward nuclear pyknosis, fragmentation of the cytoplasm, a condensation of the Golgi material to a small thick-walled hollow sphere and a decrease in mitochondria. It is our belief that the regressive non-granular basophiles in both the normal and castrated rats revert to chromophobes.

Anterior pituitaries of rats castrated for 3 months. Some years ago it was reported by Ellison and Wolfe (12) that in female rats castrated for short periods of time many of the enlarged basophiles were degranulated, but that after longer periods of castration the degranulated forms became relatively scarce. Because of these findings, the pituitary glands of 8 female rats castrated 90 days previously were studied and compared with those of the 15-day castrates already considered. It was found that a few degranulated basophiles with abundant mitochondria were present, but they were much less numerous than in the anterior lobes of the 15-day castrates. In con-

[3] Previous studies (11) indicate that the average level of basophiles in the anterior lobes of immature female rats castrated for 15 days is 15.8 per cent.

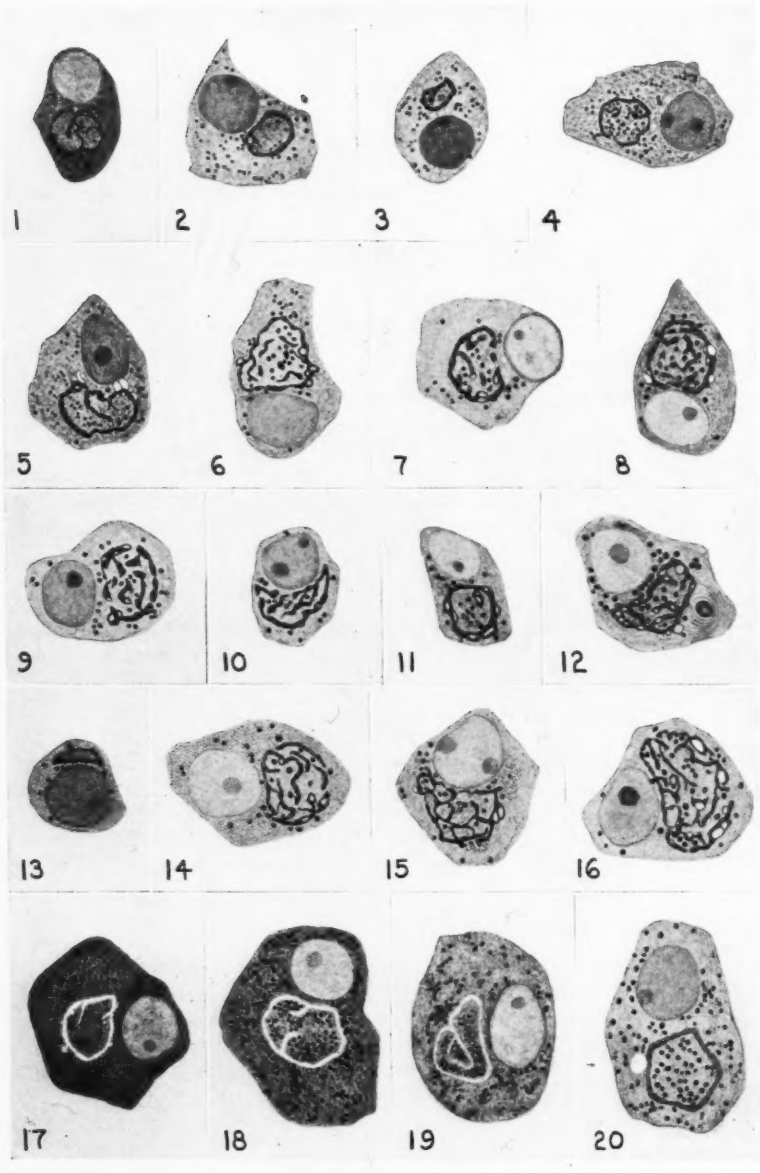

Fig. 1. GRANULAR BASOPHILE WITH large Golgi apparatus and scant mitochondria. Basophilic granules are grayish and the mitochondria black. ×1850. Fig. 2. NON-GRANULAR BASOPHILE showing abundant mitochondria and the Golgi apparatus. ×1850. Fig. 3. SMALL, NON-GRANULAR BASOPHILE. The Golgi apparatus is small and compact. ×1850. Fig. 4. PARTIALLY DEGRANULATED BASOPHILE with a moderately large Golgi apparatus and an abundant number of mitochondria. ×1850.

Fig. 5. PARTIALLY DEGRANULATED BASOPHILE. Note the abundant mitochondria and the enlarged Golgi apparatus. ×1850. Fig. 6. AN ALMOST COMPLETELY DEGRANULATED BASOPHILE. Note the large, com-

trast, there were many heavily and moderately granulated cells; in the latter, mitochondria were often numerous. Castration cells were also abundant.

It is well established that castration in the rat leads to the storage of a gonadotropic hormone in the basophiles of the anterior pituitary. These cells are greatly increased in size, number, and granular content. In several papers concerned with the cytology of the anterior pituitary gland of castrated rats, Severinghaus (7, 13, 14) has emphasized the presence of many large granular basophiles characterized by enlarged Golgi bodies and by a scant number of mitochondria. In contrast, a scarcity of degranulated basophiles was noted. The same author has consistently associated enlargement of the Golgi apparatus with elaboration of secretion, a view which is held to be true for glandular cells in general (15). He has also related an increase of mitochondria to the release of secretion or degranulation. These interpretations appear to be supported by our observations and are in our opinion correct. On the basis of his observations Severinghaus believes that, in the rat, castration results in a storage of hormone in the basophiles of the anterior lobe and that there is little evidence to indicate an increase in the discharge of secretion.

Numerous degranulated and partially degranulated basophiles with an abundance of mitochondria, in addition to the many large granular basophiles, were found in the anterior lobes of the 15-day castrates. This has led us to the view that, although castration does result in a pronounced storage of hormone in the basophiles, there is also an appreciable release of hormone from these cells, especially in the rats recently castrated. Using the cytologic criteria mentioned above, it seems likely that the degranulated basophiles with abundant mitochondria, as illustrated in figure 20, had recently discharged their secretory product. In many of these degranulated basophiles the Golgi bodies were also quite large. This has led us to consider the possibility that such cells were elaborating secretion and releasing it immediately, although it is also possible that they had recently become degranulated and that the enlarged Golgi bodies had not yet regressed from the size attained when the cells contained granules. The fact that degranulated basophiles were more numerous in the anterior lobes of the recently castrated rats than in those of rats castrated for longer periods would suggest that the rate of discharge of secretion is greater in ovariectomized rats early in the period following castration than it is after longer intervals of time.

There seems to be close agreement between our findings in the rat and those on the anterior pituitary of a post-menopausal woman (considered to be a physiological

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plicated Golgi apparatus and the small clear vesicles in its meshes. ×1850. Fig. 7. DEGRANULATED BASOPHILE with a moderately large Golgi apparatus. ×1850. Fig. 8. AN ALMOST COMPLETELY DEGRANULATED BASOPHILE with a large complicated Golgi apparatus. ×1850.

Fig. 9. DEGRANULATED BASOPHILE. Note the 'nodular' thickening of the strands of the enlarged Golgi apparatus. ×1850. Fig. 10. SMALL CHROMOPHOBE with a large Golgi apparatus, capping the nucleus. ×1850. Fig. 11. SMALL CHROMOPHOBE with a relatively large, rounded Golgi apparatus. Note the clear vesicles. ×1850. Fig. 12. LARGE CHROMOPHOBE with hypertrophied Golgi apparatus, abundant mitochrondria, and a 'Nebenkern.' ×1850.

Fig. 13. MEDIUM-SIZED EOSINOPHILE well filled with granules. The granules are shown as clear circles. X1850. Fig. 14. LARGE, PARTIALLY DEGRANULATED EOSINOPHILE. The Golgi apparatus is hypertrophied. ×1850. Fig. 15. AN ALMOST COMPLETELY DEGRANULATED EOSINOPHILE. The remaining granules are arranged as small clumps. The Golgi apparatus is hypertrophied and contains clear vesicles. ×1850. Fig. 16. LARGE CHROMOPHOBE with a markedly hypertrophied Golgi apparatus. Note the large clear vesicles and the abundant mitochondria. ×1850.

Fig. 17. LARGE BASOPHILE well packed with granules from a castrated rat. The granules are grayish and the mitochondria black. The negative image of the Golgi apparatus is shown. ×1850. Fig. 18. LARGE BASOPHILE WITH FEWER GRANULES, from a castrated rat. Note the extremely abundant mitochondria and the negative image of the Golgi apparatus. ×1850. Fig. 19. PARTIALLY DEGRANULATED BASOPHILE with abundant mitochondria, from a castrated rat. ×1850. Fig. 20. LARGE DEGRANULATED BASOPHILE from a castrated rat. Note the abundant mitochondria and the Golgi apparatus which was stained positively with aniline blue. ×1850.

These cells were drawn by Miss Alice P. Schafer.

castrate), by Severinghaus (14). He found an increase in the numbers of vacuolated cells, but more pertinent to the present discussion was the presence of many sparsely granulated basophiles in which the Golgi apparatus was enlarged and the mitochondria abundant. These findings were considered by him as indicative of an increased secretory activity. Furthermore, Kirkman (16) found that the outstanding effect of ovariectomy on the cytology of the guinea pig pituitary was an increase in the granular basophiles, but he also found there was an increase in the number of non-granular basophiles which were characterized by increased numbers of mitochondria.

Certain physiological findings also indicate that the anterior pituitaries of castrated human beings and rats release gonadotropic hormones into the blood stream. It is well known that large amounts of this factor, which seems to be chiefly follicle stimulating, is found in the blood and urine of castrated and post-menopausal women. In parabiosis experments it has been demonstrated by Witschi and Levine (17) that the anterior lobes of castrated rats release sufficient gonadotropic hormone to stimulate marked follicular growth in the ovaries of the hypophysectomized partners. Although a correlation of cytological and physiological data must be made with caution, it seems that the cellular changes described by us in the rat, and by Severinghaus in women, supply at least a qualitative cytologic basis for the release by the anterior lobes of castrated female rats and of castrated women of a gonadotropic factor which seems to be chiefly follicle stimulating. The physiological data is usually interpreted as indicating a hypersecretion of the follicle stimulating factor from the castrate pituitary, although Severinghaus (14) has rightly pointed out that a surplus of circulating gonadotropic hormone could probably be expected in castrates even if release continued normally. Our findings indicate the release of hormone from many basophiles of the castrate rat pituitary but because of inadequate control material from normal rats, a definite statement concerning a cytological basis for the release of excess quantities of hormone cannot be made.

Anterior pituitaries of rats receiving diethylstilbestrol. The anterior lobes of the normal and castrated rats receiving the synthetic estrogen were identical in their structural characteristics.

The glands of rats which had received 1 mg. daily of diethylstilbestrol for periods of 3 and 5 days well demonstrated the effects of estrogen administered over a short period of time. The eosinophiles in the anterior lobe of rats injected for 3 days were practically unchanged, while those of the rats treated for 5 days showed a mild degree of degranulation. Since the reaction in the eosinophiles was so much more pronounced in animals injected for longer periods the changes in these cells will be described later in this report when the anterior lobe of such rats are considered.

Profound and widespread changes were found in the basophiles. Granular, partially degranulated and non-granular basophiles were found in the glands of rats injected for 3 days. The granular cells, while not numerous, were similar in appearance to those found in the controls, although the Golgi apparatus tended to be somewhat larger. This body often lost the characteristic form of an irregular sphere and appeared to break up into a mass of anastomosing osmiophilic strands, as described previously by Severinghaus (7). Often this mass was itself more or less spherical, but in many cells it was irregular in form and sometimes was pressed against the nucleus. The mitochondria were not as abundant in the heavily granulated basophiles as they were in those in which the granular content was reduced.

The partially degranulated basophiles varied considerably in appearance. A considerable number of these cells were similar to those often seen in the non-castrated control rats (fig. 4). The Golgi apparatus appeared as a section through an irregular

and not enlarged sphere and the mitochondria were quite numerous and distributed fairly evenly throughout the well.

There were many partially degranulated basophiles in which the Golgi apparatus was markedly hypertrophied (fig. 5, 6, 8). The enlarged Golgi bodies were made up of osmiophilic strands which usually anastomosed, but sometimes were separated. The mass of Golgi material tended to be spherical and to lie free in the cytoplasm, but in some cells it was pressed against the nucleus. The clear vesicles associated with the Golgi material were abundant and often large. As was to be expected, other partially degranulated basophiles were observed in which the Golgi apparatus was intermediate in size and form between the two extreme types noted above.

In the degranulated basophiles the Golgi apparatus varied markedly in size and appearance. In many of the cells the Golgi body was rather small and spherical, similar to that in figures 2 and 3; in such cells the abundant mitochondria were scattered throughout the cell. In other cells the Golgi apparatus showed pronounced hypertrophy (fig. 7 and 9). The Golgi material was often separated into anastomosing strands and at certain points these showed a tendency to become concentrated into small nodules which were often connected by thin threads of osmiophilic material (fig. 9). The nucleoli in these cells were generally large and prominent, while mitochondria were usually, but not always, concentrated in the Golgi region. Interposed between the degranulated basophiles in which the Golgi apparatus was enlarged, and those in which it was not, were cells of all degrees of intermediate types.

An analysis of the cytological changes which occurred in the basophiles was difficult because of certain changes which occurred in the chromophobes. In many of these cells the Golgi apparatus was hypertrophied and the mitochondria were more abundant and prominent. In a few cells the Golgi material formed a hollow, irregular sphere (fig. 11) but in most of the cells (fig. 10) a larger and more complicated apparatus was found. In both types, small clear vesicles in the Golgi material were frequently observed. These altered chromophobes appeared to us to be the cells which Severinghaus described and classified as new basophiles, recently differentiated from chromophobes in the anterior lobes of rats receiving small or moderate amounts of estrone (18). Our observations indicated that these modified chromophobes became larger and that it was was impossible to differentiate them from degranulated cells which arose as the result of basophilic degranulation. There was no conclusive evidence, however, that they actually released basophilic secretion.

Many of the changes depicted above, and which followed a short injection period of synthetic estrogen, have previously been described by Severinghaus (14, 19) who used natural estrogens. He found that injections of estrone over a short period of time caused basophilic degranulation, Golgi hypertrophy, an increased prominence of the mitochondria and the differentiation of new basophiles from chromophobes. Previously, Nelson (20) and Halpern and D'Amour (21) had also reported basophilic degranulation and an increase of mitochondria following estrogen administration.

In analyzing his findings Severinghaus (14) concluded that estrogen stimulated secretory activity in the basophiles. The degranulated cells with the enlarged Golgi bodies and prominent mitochondria were considered to be producing secretion after they had become degranulated, and to be releasing the secretion as rapidly as it was formed. In accordance with this view it is thought that the large degranulated basophiles shown in figures 7 and 9 were producing and releasing secretion while the degranulated basophiles in figures 2 and 3 had discharged their secretion and were not producing new secretion rapidly, although the two small vesicles in the Golgi material of the cell in figure 2 may possibly be considered evidence of secretory activity

(8, 9). Although it is thought that most of the degranulated basophiles of the type illustrated in figures 2 and 3 revert directly into inactive chromophobes, it seems possible that a basophile, even though it is in the phase of degranulation may be capable of producing secretion if the proper stimulus becomes operative. Although we realize that an interpretation concerning the relation of the cytological appearance and functional activity of cells must be considered as tentative, it is thought that the above analysis does correlate well with the cellular changes noted.

In rats injected for longer periods of time (up to 15 days) an intensification of the cellular changes in the anterior lobe occurred. An outstanding feature was the widespread degranulation of the eosinophiles (fig. 14, 15). The degranulating cells were usually somewhat swollen, the granules becoming less closely packed and often themselves appearing to undergo a slight degree of swelling. Moreover, it was found, with considerable constancy, that the granules in the larger partially degranulated cells stained more lightly than usual. In the almost completely degranulated cells the remaining granules tended to be arranged in small clumps (fig. 15).

Degranulaton in the eosinophiles was almost invariably accompanied by hypertrophy of the Golgi apparatus which often became so large that it occupied a major portion of the cytoplasm (fig. 14, 15). Frequently it was more or less semilunar in shape (fig. 15) and lay close to the nucleus or in contact with it; in some cells it was more rounded (fig. 14) and occupied a position somewhat removed from the nucleus. Although we agree with Severinghaus that there are normally fairly constant differences in the form and position of the Golgi apparatus in the eosinophiles and basophiles of rat pituitaries, we also feel that these differences become less conspicuous or may disappear when the hypertrophied Golgi bodies in the eosinophiles and basophiles are compared in rats injected with estrogens. Small clear vesicles in the Golgi material were abundant, and in some instances became quite large.

Degranulation was generally accompanied by an increase in the number and size of the mitochondria, although in some cells this change was not conspicuous. In cells in which a considerable number of granules remained (fig. 14) the mitochondria were scattered throughout the cell but as the cell approached more complete degranulation, there was a tendency for the mitochondria to be concentrated in the region of the hypertrophied Golgi apparatus (fig. 15). Since the degranulating eosinophiles were characterized both by Golgi hypertrophy and an increased prominence of the mitochondria, it seems probable that the diethylstilbestrol stimulated these cells not only to release their stored hormone but at the same time to produce secretion.[4] However, since many cells became completely degranulated, the discharging process must have progressed more rapidly than did the production of hormone.

Granular basophiles were not observed in these glands, and the only non-granular basophilic cells which could be identified with certainty were those in which there was a small Golgi apparatus and variable numbers of mitochondria (fig. 2, 3).

These pituitaries were characterized by tremendous numbers of small, medium and large non-granular cells. A majority of these, particularly the medium and large cells, were characterized by Golgi hypertrophy (fig. 12, 16) and by the presence of prominent mitochondria which were often concentrated in the region of the Golgi apparatus. Both the observations of the cytological characteristics in this study, and cell counts made in previous studies by Wolfe and Chadwick (22), indicate that many of these non-granular cells appeared as the result of degranulation among the eosino-

[4] The fact that there is cytologic evidence of production of secretion in these cells which are undergoing degranulation would suggest a mechanism for the conservation of the hormone content of these cells. In like manner, the numerous mitotic figures found in the eosinophiles of rats receiving estrogen (22) suggests an attempt to maintain these cells at a normal level under conditions which otherwise induce a decrease in these cells.

philes and basophiles. It is also considered quite possible that many of the large degranulated cells with large Golgi bodies and prominent mitochondria were the result of the direct initiation of secretory activity in the chromophobes. In these pituitaries we have found it impossible to differentiate, on the basis of the extremely variable size and form of the Golgi apparatus, the non-granular cells which originated from basophiles from those which arose as the result of degranulation of the eosinophiles. A similar conclusion has recently been reached by Severinghaus (14).

It is a question just how these cells should be classified. They are non-granular and are, in the strict sense, chromophobic. However, the usual conception of a chromophobe is a small non-granular cell with a small Golgi body, few mitochondria and scant cytoplasm. Therefore, as has already been stated by Severinghaus (14), the cells under discussion are not typical chromophobes because they present cytological evidence of producing and releasing hormone at a greatly increased rate. On the other hand, it is not feasible to classify them as degranulated eosinophiles or degranulated basophiles because the two types cannot be differentiated from each other. They might possibly be termed 'active' chromophobes while the small, inactive cells, characterized by scant cytoplasm, a small Golgi apparatus and few mitochondria, could be classified as either 'typical' or 'inactive' chromophobes. But even this is unsatisfactory because, in rats injected with estrogen, the small inactive chromophobes may give rise to larger cells characterized by a large Golgi apparatus and abundant mitochondria. This results in such a host of intermediate variants that a positive designation of individual cells becomes almost impossible. At the present time we can offer no satisfactory solution for this problem of terminology.

Two other observations, the significance of which is not clear, warrant brief mention. The anterior lobes of 2 rats, each of which had received 100 mg. of diethylstilbestrol during a period of 15 days, showed a greater degree of mitochondrial hypertrophy in the large active chromophobes and partially degranulated eosinophiles than was usually found. In a few cells in the chromated tissue large spherical bodies which were considered to be markedly hypertrophied mitochondria were noted. Usually they stained with a normal intensity but there was a tendency for some to take a paler fuchsin stain. In occasional cells rounded bodies of similar size which stained a dull yellow or a brownish yellow were found. Transitional stages between the large spherical bodies which stained with fuchsin and those which took a yellow stain could be found. Although there is no positive evidence, we are inclined to the view that these cytoplasmic structures, both those which stained red and those which stained yellowish, represented altered mitochondria. In the osmic-treated tissues large black spherical bodies were occasionally seen; it is considered probable that these were the same hypertrophied bodies noted in the chromated tissues. Large spherical masses of mitochondrial origin have been reported previously by Schooley (23) in the basophiles of the pigeon pituitary. Although their significance is not clear, they appear to us to be in some way associated with intense cellular activity.

During this study fine filaments were frequently noted in the cytoplasm of the anterior lobe cells. Often these were arranged in rounded masses and formed the so-called 'Nebenkern' (See Bowen, 15, for a review of the general literature and Kirkman, 16, for literature pertinent to the anterior lobe). These bodies occurred most often in large active chromophobes (fig. 12) or in the agranular portion of the cytoplasm of partially degranulated eosinophiles. The 'Nebenkern' seemed to consist of a variable number of circular cytoplasmic filaments arranged around a central mass or core (fig. 12). The origin of the central mass appeared to be variable. It was often formed by one of the large spherical bodies just described which stained either red or dull yellow. Infrequently, the circular filaments were found around a small clump of eosinophilic

granules and in some instances they were arranged around a small dense mass of apparently ordinary cytoplasm. In chromated sections the filaments stained dull blue; in osmic preparations both the central mass and the filaments were often, but by no means invariably, osmiophilic. There seems to be considerable evidence which indicates that these structures are fixation artifacts (15) and our observations support this view. For instance they occur much more frequently in tissue fixed in Champy's fluid than in material fixed in Regaud's solution. They are also more abundant in the anterior lobes of rats treated with estrogen than in those of normal animals, presumably because of the presence of increased numbers of large active chromophobes in which they are most often found.

DISCUSSION

In castrated or post-menopausal women and in castrated rats, the physiological and cytological findings on the anterior lobe appear to be in good agreement. Functional studies indicate that such pituitaries release considerable amounts of gonadotropic hormone which is predominantly follicle stimulating, although some luteinizing hormone may be present (24). Cytological observations strongly suggest that the basophiles are releasing secretion, in spite of the fact that the predominant effect of castration is storage of basophilic secretion. These findings support the idea that the basophiles may be the source of the follicle stimulating factor.

Following the administration of estrogen, certain discrepancies between functional and structural studies arise. The results of several types of physiological experiments have led to the general belief that estrogen 'inhibits' the function of the anterior lobe, while cytological studies usually suggest a stimulation of both hormone release and production. Thus, it is well known that estrogen injections will decrease the gonadotropic potency of the anterior pituitaries of rats when tested by the implant method. However, as has already been noted by Severinghaus (14), this is due to the fact that the estrogen stimulates the release of the hormone from the gland and a decrease in potency is the result. This is supported cytologically by pronounced degranulation of the basophiles and eosinophiles and an increase in the prominence of the mitochondria in the glands of the treated rats. Severinghaus has concluded that these experiments do not indicate an inhibition of anterior lobe activity, but that there is actually a stimulation of hormone release. In this particular type of experiment, therefore, cytological and physiological findings can be brought into good agreement.

There are certain physiological findings, however, which indicate an actual inhibition of anterior lobe function by estrogen. Thus, administration of sufficient amounts of estrogen to castrated or post-menopausal women will either abolish or lower the amounts of gonadotropin in the urine (25). In parabiotic rats, injection of estrogen into the castrated partner prevents the ovarian hypertrophy in the non-castrated twin (26). Since all available evidence indicates that the gonadotropin released by the anterior lobe of castrates, both women and rats, is chiefly follicle stimulating, these findings have been accepted as evidence that estrogen inhibits the production and release of FSH; see Fevold (27) for review of literature. Actually, such observations taken alone indicate only an inhibition of release of hormone. However, since castration in both the rat and human female results in an increase in the number and size of the basophiles, and since these cells are usually associated with the production of FSH, the fact that estrogen will prevent this reaction may well indicate that estrogen prevents the production as well as the release of FSH.

It seems generally agreed that estrogen stimulates the release of a luteinizing hormone (LH) from the anterior lobe which transforms follicles into corpora lutea; see Fevold (27) for review of literature. More recent studies of Astwood (28) indicate that LH itself does not maintain the activity of the corpora lutea, but that the luteal function is stimulated and maintained by an additional anterior lobe factor, the release of which is stimulated by estrogen. This factor, which he designated as luteotropin, has recently been identified as prolactin by Evans and his group (29). Physiological data suggest, therefore, that estrogen not only inhibits the production and release of FSH but that it stimulates the release of LH and the luteotropic factor. Although this view is supported by considerable evidence, it has proven difficult to harmonize cytological findings with this concept. The chief difficulty seems to be the fact that following estrogen administration, cytological evidence of inhibition is not readily apparent.

Although estrogen stimulates marked secretory activity in the basophiles, and these cells are usually thought to produce FSH, we do not know of any evidence, with the exception of a single report by Lane (30), that estrogen brings about the release of such a factor. Actually, as we have just indicated, most evidence indicates exactly the opposite. Although a satisfactory explanation for this discrepancy is not known, two suggestions may be offered. *a*). It is possible that FSH is released after estrogen injections and that another factor is discharged simultaneously which inhibits its action. Indeed, the recent results of Fevold and Fiske (31) indicate that LH may exert such an action; some years ago Evans and his associates (32) reported the isolation of an anterior lobe factor which inhibited the action of FSH. *b*). Although the basophiles may produce and release FSH under certain conditions, it is considered possible that under the influence of estrogen, they may produce and release an entirely different factor. Either of the above possibilities would explain the apparent disharmony between physiological and cytological observations.

Estrogen also stimulates the eosinophiles, causing them to degranulate and, judging by the hypertrophied Golgi bodies, to produce secretion. Since there is some evidence which links the eosinophiles to the processes of luteinization and luteal function (14, 33), and since estrogen apparently stimulates the release of LH and the luteotropic factor, cytological and physiological observations appear to be in harmony. However, even here a discrepancy appears. Although estrogen induces marked hypertrophy of corpora lutea in mature female rats (34), it has been demonstrated by Merckel and Nelson (35) that these undergo regression at the end of about three weeks in spite of continued treatment. These authors have suggested that the regression is due to secretory exhaustion of the anterior lobe. However, we have never observed any cellular changes in the anterior pituitary glands of rats injected for three weeks, or much longer periods, which we feel would suggest a basis for functional exhaustion. Furthermore, Astwood (28) has found that luteal function cannot be maintained indefinitely in spite of the continued injection of the luteotropic factor itself. This would suggest that the functional life of the corpus luteum is determined by some inherent factor rather than functional exhaustion of the anterior lobe.

At the present time there are still many apparent contradictions between the cytological and physiological findings on the anterior pituitary. However, there is also harmony in many instances and it seems likely that as new physiological facts become known, and as certain refinements in cytological technic are made, many of the discrepancies between the interpretations of the results of the two methods will disappear.

SUMMARY

Normal and castrated immature female rats received various amounts of diethylstilbestrol for periods of time ranging from 2 to 15 days. Control observations were made on uninjected, normal, immature females and in immature females castrated for 15 and for 90 days.

Administration of diethylstilbestrol for short periods induced pronounced degranulation of basophiles in the anterior pituitary. Many of the degranulated basophiles were characterized by hypertrophy of the Golgi apparatus and by an increase in the number of the mitochondria; in others, the Golgi body was not enlarged, but mitochondria were abundant. Marked changes were also observed in the chromophobes, many of which exhibited an enlargement of the Golgi apparatus and an increase in the prominence of the mitochondria. Changes in the eosinophiles, while they did occur in rats injected for short periods of time, were more pronounced after the longer periods of injection. These cells underwent degranulation accompanied by enlargement of the Golgi body and by an increase in the number and size of the mitochondria. The changes induced by diethylstilbestrol in the anterior lobe are identical to those produced by the naturally occurring estrogens.

Castration for 15 days resulted in a conspicuous increase in the number and size of the granular basophiles. However, many partially and completely degranulated basophiles were also seen. These were generally characterized by abundant mitochondria. In rats castrated for 90 days there was much less evidence of basophilic degranulation.

The correlation of physiological and cytological findings on the anterior pituitary were discussed.

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NOTES AND COMMENTS

VARIATIONS IN ESTROGEN CONTENT OF URINE OF FEMALE RABBITS

WHILE CONSIDERABLE STUDY has been made of the estrous cycle of the rabbit,[1,2,3] practically no attempt has been made to elucidate this phenomenon by a consideration of variations in urinary estrogen. Pincus, Wheeler, Young, and Zahl[4] and Pincus and Zahl[5] have concluded that the rabbit excretes little or no estrogen in the urine. Having had success in similar studies on rats,[6] the author decided to apply more recent refinements of the assay methods to rabbit urine, in hopes of extending our knowledge in this field.

Method. The urine was collected from adult female albino rabbits about 8 months of age, all of whom had had at least one litter. The rabbits were catheterized at 8 A.M., 2 P.M., and

TABLE 1. ESTROGEN EXCRETION VALUES IN RABBITS

Source of Urine Sample	R.U./Liter Urine	Source of Urine Sample	R.U./Liter Urine
Males	0	*Females*, directly after parturition	
Females, stimulated to ovulation by coitus, and urine for the 24 hours following pooled	5	1– 5 days (collected daily)	5
		6–30 days (collected in 5-day pools)	5
Females, virgin, aged 8 months, bred in Michigan		*Pseudopregnant females* (July–August)	
January–February	2	1– 5 days	10
March–April	2	6–10 days	20
May–June	4	11–15 days	25
July–August	4	16–20 days	10
September–October	2	21–25 days	5
November–December	2	26–30 days	5
Pregnant females (during July–August)			
1– 5 days	10		
6–10 days	40		
11–15 days	100		
16–20 days	180		
21–25 days	50		
26–32 days (term)	10		
33–37 days	5		

8 P.M. daily, a total of about 25 cc. of urine per rabbit per day being obtained. Rabbits were mated and the urine collected from females in successive arbitrary 5-day pools to produce an average sample of reasonable size. The estrogen values for pregnancy were determined on these samples. Samples were collected in daily pools representing the first 5 days after parturition, in an effort to get pools of urine representing stages of a normal estrous cycle, but all of the pools assayed approximately the same, indicating that no short cycle occurred at the time. Subsequent pools, representing 5-day periods were collected, in a further effort to get

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pools of urine representing corresponding stages of a possible normal estrous cycle. This process was continued for 30 days, after which it was felt that the phases of the cycles of different animals were sufficiently mixed to give misleading results. Similarly, samples were collected in 5-day pools from females, dating from the inception of pseudopregnancy. In all cases, the amount of urine collected was measured, in the hope that it might yield information on water metabolism during the period under observation. The urine was all collected during July and August, except as otherwise noted, and was kept in glass-stoppered bottles at 4° C. for not longer than one week. It was assayed by the methods of MacCorquodale Thayer, and Doisy,[7] and the results of the assays were recorded in table 1 in rat units per

TABLE 2. URINE EXCRETION VALUES IN FEMALE RABBITS

Source of Urine Sample	Average cc. of Urine/Day	Source of Urine Sample	Average cc. of Urine/Day
Monthly pools from virgin females		*Pseudopregnant females*	
May–August	20	1–10 days	22
September–April	30	11–20 days	26
Pregnant females		21–30 days	23
1–10 days	24	*Females after parturition*	
11–20 days	28	1– 5 days	22
21–32 days	30	6–10 days	22
		11–15 days	22
		16–20 days	22

liter. This data may be calculated to rat units per day by means of table 2, which gives the data on the amounts of urine excreted.

Summary. Results in general seem to confirm existing knowledge regarding the sexual characteristics of the rabbit. No estrogen was found in the male urine. It is evident that small but definite amounts of estrogen are always present in female rabbit urine, and that these amounts seem to increase slightly toward mid-summer. Whether this is due to richer diet, exposure to greater radiation, or to other factors, is uncertain, but it seems doubtful if the corresponding rise in the volume of urine excreted in summer is due to any but purely obvious reasons. The lesser concentration of estrogen in winter is doubtless associated with the period of relative anestrus at that time.

Studies of the urine immediately after parturition would indicate that, at that time at least, there is no evidence of cyclic variations. This fact seems well supported by histological evidence.[8] Moreover, careful studies over the period of 72 hours immediately following coitus fail to show any variation corresponding with the known incidence of ovulation.

There is a marked rise in the urinary estrogen from pregnant does, which reaches a peak about midterm, in this respect resembling the corresponding data for the mare[9] and varying from that for the human species[10] in which the peak of excretion is at term. This may explain why Pincus et al.[1] found only small amounts of estrogen from the 27th to the 30th days of pregnancy in the rabbit. There is a similar rise in the excretion values in the pseudopregnant rabbit, although the magnitude is only one-tenth of that in the pregnant animal, and it falls off earlier than the drop of the urinary estrogen occurs in the pregnant animal. Both the size and the duration of this rise enhance the comparison made by Hammond[8] and others of pseudopregnancy with the estrous cycle of other animals.

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